

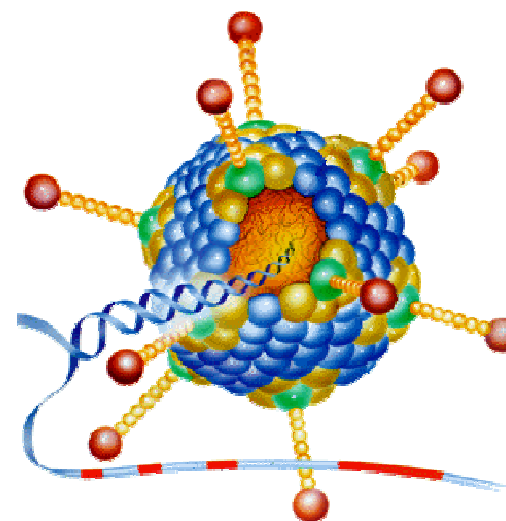
Vaccination has been one of the most important interventions designed to prevent disease to be employed on a worldwide basis. Adenoviral vector has been extensively studied as a vaccine platform because of its ability to induce potent cellular and humoral immunity. Consequently, the market requirements for adenoviral vectors are increasing, creating a need for new methodologies for large-scale production of concentrated vectors with warranted purity and efficacy. This work contributes for the improvement of the state of the art of adenoviral vectors production process.

Tiago Bruno Ferreira

Adenovirus as a Biopharmaceutical: Optimisation of vector production

# Adenovirus as a Biopharmaceutical

*Optimisation of vector production*

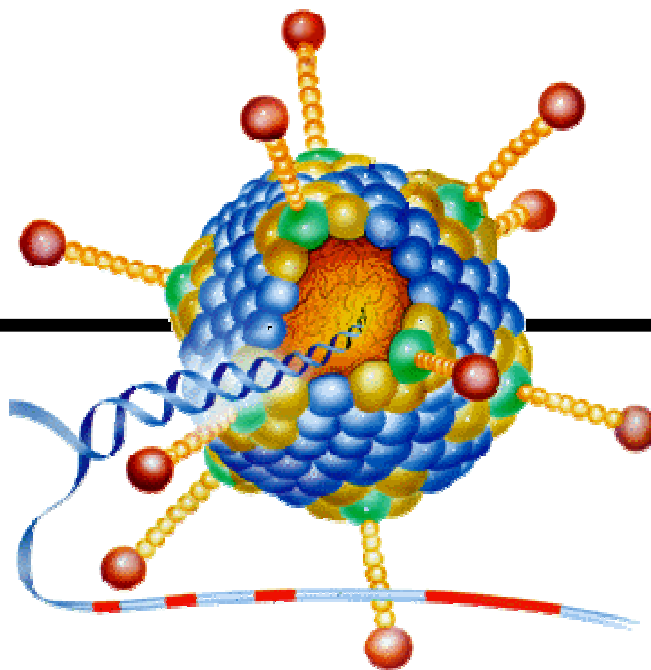


Tiago Bruno Ferreira



Dissertation presented to obtain a Ph.D degree in Chemical Engineering at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa

Oeiras, March 2007



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Optimisation of vector production

By

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Universidade Nova de Lisboa  
Oeiras, March 2007

## Adenovirus as a Biopharmaceutical: Optimisation of vector production

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Back: Electron microscope picture of adenovirus particles (up image) and microscope picture of 293 cells infected with adenovirus vectors (back image).

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## Foreword

The present thesis dissertation is the result of four years of research at the Laboratory of Animal Cell Technology at ITQB/IBET (Portugal) under Dr. Paula M. Alves and Prof. Dr. Manuel J. T. Carrondo Supervision.

This thesis intends to explore the limiting factor on adenovirus vector production at high cell densities aiming the understand and improvement of the current inefficient process of adenovirus vector production in order to respond to the increasing demand of methodologies for the production in large scale of concentrated vectors with warranted purity and efficacy.

## Acknowledgements

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A very special thanks to my supervisor Dr. Paula Marques Alves, who truly made a difference in my life. It was under her tutelage that I developed a focus and became interested in the animal biotechnology field. She provided me with direction, suggestions and support. It was through her persistence, understanding and kindness that I completed this work. I doubt that I will ever be able to convey my appreciation fully. Thank you very much.

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I would also like to thank my parents and brother for the support they provided me through my entire life without whose love and encouragement I would not have finished this thesis.

I must give immense thanks to my patient and loving wife Alexandra and our daughter Carolina, who have been a great source of strength all through this work, especially during those times when the research came first. Their love and support was of immeasurable value to me.

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## Abstract

Adenovirus vectors (AdV) have attracted considerable interest over the past decade, with ongoing clinical development programs for applications ranging from replacement therapy for protein deficiencies to cancer therapeutics to prophylactic vaccines. In fact, in the period 1989-2005, over 300 clinical trials were conducted using adenoviruses worldwide, adenovirus type 5 (Ad5) being the vector of choice. Consequently, considerable product process, analytical and formulation development has to be carried out in order to respond to the constantly increasing market requirements for AdV.

One of the major restrictions on AdV production is the so-called "cell density effect", i.e., a drop in cell specific productivity concomitant with increased cell concentration at infection (CCI) above  $1 \times 10^6$  cells/ml. This limitation on AdV production has to be fine-tuned in order to obtain higher AdV volumetric productivities; this will be the subject of study and development in this thesis.

In Chapter I, a state of the art on recent developments and approaches of AdV production and purification, including successes and failures in veterinary clinical applications to date, is presented.

In Chapter II, the cell density effect is explored. Thus, AdV production at cell densities higher than  $1 \times 10^6$  cell/ml is evaluated by comparing two different serum free media (CD 293 medium from Invitrogen and EX-Cell medium from JRH). The results show that while EX-Cell medium allows the higher maximum cell number ( $7.5 \times 10^6$  cell/ml), CD 293 is the best option for AdV production at high cell densities, allowing infection at CCI of  $2 \times 10^6$  cell/ml without significant losses in terms of cell specific productivity.

Nevertheless, although CD 293 medium allows cell growth up to  $5.5 \times 10^6$  cells/ml, when infection is performed at CCI of  $3 \times 10^6$  cell/ml a significant drop in cell specific productivity is observed. This phenomenon was tentatively attributed to nutrient limitations and/or accumulation of toxic byproducts. These byproducts (e.g. lactate) are also associated with an increase on osmolality. In addition, the possible need to develop a refeed strategy must take into account the consequent increase in osmolality. Thus, the effect of osmolality on cell growth and AdV production was also evaluated in this Chapter. The capacity of 293 cells to produce AdV showed a good tolerance to increases in osmolality.

In chapter III, the limiting factors on AdV production at high cell densities are discussed. In Part 1, amino acids, glucose, lactate and ammonia metabolism are evaluated in non-infected and infected cells at CCI of 1 and  $3 \times 10^6$  cells/ml. Threonine, glutamine and glucose were observed to be totally consumed on AdV production at CCI of  $3 \times 10^6$  cells/ml. However, no improvements on AdV production were obtained after addition of these nutrients at infection time. On the other hand, ammonia was observed to be at a toxic concentration for cell growth at the time of infection at this CCI. Thus 293 cells were adapted to grow in glutamate supplemented medium instead of glutamine, a non-ammoniagenic medium. With this strategy a significant decrease on ammonia cell specific production rate was obtained with an increase of 1.8 times on the AdV volumetric productivity when infection was performed at CCI of  $3 \times 10^6$  cells/ml. Therefore, ammonia is an important factor on AdV production at high cell densities. In order to understand the reasons for such inhibition, in Part 2 ammonia inhibition mechanisms are discussed by comparing both ammoniagenic (glutamine supplemented) and non-ammoniagenic media. The results show that Intracellular pH ( $pH_i$ ) is severely affected at high ammonia concentration, as expected. Besides, these changes on  $pH_i$  were observed to play an important role on cell viability and AdV DNA stability,

decreasing the time during which cells are producing AdV and the availability of AdV DNA for encapsulation, consequently decreasing the amount of bioactive AdV particles produced.

In Chapter III, by adapting the cells to non-ammoniogenic medium, an 1.8 fold increase on AdV volumetric productivity at CCI  $3 \times 10^6$  cell/ml was obtained, although the “ideal” situation would have been an increase of 3 times at this CCI; thus, ammonia is an important parameter to be considered for infection at high cell densities, but not the only one. Thus, in Chapter IV, the physiological state of the cells at the time of infection at different CCIs is evaluated by following the cell cycle. Therefore, in Part 1, the evaluation of the cell cycle through the cell culture is shown. At the optimal CCI (CCI of  $1 \times 10^6$  cells/ml) the amount of cells at the S phase of the cell cycle was observed to be in its maximal and to decrease with the increase on the cell density. In addition, by infecting cells at the same cell concentration with different percentages of cells at the S phase of the cell cycle an increase on cell specific productivities was observed with the increase on the percentage of cells at the S phase of the cell cycle, suggesting that the cell specific productivity is cell cycle phase dependent at infection time. To clarify this question, in Part 2, 293 cells were synchronised at each phase of the cell cycle with chemical compounds and infected at the same CCI. The higher cell specific productivity was obtained when cells were synchronised at the S phase of the cell cycle, confirming our previous hypothesis. Thus, since the physiological state of the cells is an important parameter to consider at the time of infection on AdV production, a strategy avoiding the use of chemical inhibitors was developed by using temperature shift. With this strategy a synchronisation of 57% of the cells at the S phase of the cell cycle by just decreasing the temperature to 31°C during 67h and increasing it again to 37°C for 72h was obtained. By infecting these synchronised cells, an increase of 7.3 times on the cell specific productivity was obtained. However, due to losses on cell

viability and the higher incubation times necessary to achieve S phase cell synchronisation, more studies are still required to fine tune the process.

In the Annexes two additional studies are presented. In Annex A, the importance of infection kinetics, such as the MOI (Multiplicity Of Infection) and HPI (Hours Post Infection) on AdV production process optimisation were evaluated. In addition, the use of serum supplemented media were compared with a serum free media in terms of cell growth and AdV production; in Annex B, the effect of adding vitamins or lipids at the time of infection at high cell densities are evaluated, no significant improvement on AdV production being obtained.

This thesis presents relevant contributions to the current state of the technology of manufacturing AdV. Both medium limitations (either lack of nutrients or excessive byproducts production, namely ammonia) and the physiological cell state condition at the time of infection were found to be valuable parameters to be considered for AdV production at high cell densities.

## Sumário

Os vectores adenovirais (AdV) têm atraído bastante interesse durante a última década, com o desenvolvimento de programas de aplicação clínica que vão desde a terapia de substituição para défices proteicos a terapias contra o cancro e vacinas profiláticas. Na realidade, no período 1989-2005, mais de 300 testes clínicos foram realizados em todo o mundo usando adenovirus, maioritariamente adenovirus do tipo 5 (Ad5), sendo um vector de excelência. Consequentemente, o desenvolvimento de um processo para produção de quantidades consideráveis de producto, analíticos e formulações têm que ser realizados de forma a dar resposta ao constante aumento dos requerimentos do mercado para os vectores adenovirais.

Uma das maiores restrições na produção de AdV é o tão chamado “efeito da densidade celular”, isto é, uma queda na productividade celular específica com o aumento da concentração celular na infecção (CCI) acima de  $1 \times 10^6$  células/ml. Esta limitação na produção de AdV tem que ser cuidadosamente analisada de forma a obter elevadas productividades volumétricas de AdV; estes vão ser o tema de estudo e desenvolvimento nesta tese.

No Capítulo I, é realizado uma revisão do estado de arte sobre os desenvolvimentos e estratégias mais recentes para a produção e purificação de AdV, incluindo sucessos e insucessos obtidos em aplicações clínicas veterinárias.

No Capítulo II o efeito da densidade celular é explorado. Desta forma, a produção de AdV a concentrações celulares superiores a  $1 \times 10^6$  células/ml é avaliada pela comparação de dois meios sem soro diferentes (meio CD 293 da Invitrogen e o meio EX-Cell da JRH). Os resultados mostram que

enquanto o meio EX-Cell permite a obtenção do número mais elevado de células ( $7.5 \times 10^6$  células/ml), o meio CD 293 é a melhor opção para a produção de AdV a elevadas densidades celulares, permitindo a infecção a uma CCI de  $2 \times 10^6$  células/ml sem perdas significativas em termos de productividade celular específica. De qualquer forma, observou-se que apesar do meio CD 293 permitir um crescimento celular até  $5.5 \times 10^6$  células/ml, a productividade celular específica é dramaticamente afectada quando a infecção é realizada à CCI de  $3 \times 10^6$  células/ml.

Este fenómeno foi atribuído à limitação de nutrientes e/ou acumulação de bioprodutos tóxicos. Estes bioprodutos (ex. Lactato) estão também associados a aumentos na osmolalidade. Adicionalmente, a possível necessidade de desenvolver uma estratégia de “refeed” deve ter em consideração o consequente aumento na osmolalidade. Assim, o efeito da osmolalidade foi também avaliado neste capítulo em termos de crescimento celular e produção de AdV. As células 293 mostraram grande tolerância aos aumentos na osmolaridade na produção de AdV.

No Capítulo III, são discutidos os factores limitantes na produção de AdV a elevadas densidades celulares. Na parte 1, o metabolismo de aminoácidos, glucose, lactato e amónia é estudado em células não infectadas e infectadas à CCI de 1 e  $3 \times 10^6$  células/ml. Observou-se que a treonina, glutamina e glucose foram totalmente consumidos na produção de AdV à CCI de  $3 \times 10^6$  células/ml. Mas não foi obtida qualquer melhoria na produção de AdV depois da adição destes nutrientes na altura da infecção. Por outro lado, foi observada uma elevada concentração de amónia na altura da infecção a esta CCI, considerada tóxica para o crescimento celular. Assim sendo, as células 293 foram adaptadas a crescer em meio suplementado com glutamato em vez de glutamina, num meio não-amóniagénico. Com esta estratégia obteve-se uma diminuição significativa na taxa específica de produção de amónia com um aumento de 1.8 vezes na productividade volumétrica de AdV quando a infecção é realizada à CCI de  $3 \times 10^6$

células/ml.

Desta forma, demonstrou-se que a amónia é um factor importante para a produção de AdV a elevadas densidades celulares. Por forma a compreender os motivos para esta inibição, na Parte 2, os mecanismos de inibição pela amónia são discutidos pela comparação de ambos os meios amóniagénico (meio suplementado com glutamina) e não-amóniagénico. Os resultados mostram que, como esperado, o pH intracelular ( $\text{pH}_i$ ) é fortemente afectado a elevadas concentrações de amónia. Por outro lado, observou-se que estas alterações no  $\text{pH}_i$  têm um importante impacto na viabilidade celular e na estabilidade do DNA dos AdV, diminuindo o tempo durante o qual as células produzem AdV e a quantidade de DNA de AdV disponível para encapsulação, diminuindo consequentemente a quantidade de partículas bioactivas de AdV produzidas.

No Capítulo III, ao adaptar as células ao meio não-amóniagénico, foi obtido um aumento de 1.8 vezes na productividade volumétrica de AdV à CCI de  $3 \times 10^6$  células/ml. No entanto a situação “ideal” seria um aumento de 3 vezes a esta CCI; deste modo mostrou-se que apesar da amónia ser um factor importante a ser considerado para a infecção a elevadas densidades celulares não é o único a ter em consideração. Assim, no Capítulo IV, o estado fisiológico das células na altura da infecção a diferentes CCIs é estudado seguindo o ciclo celular: Na Parte 1, é feito o estudo do ciclo celular durante o crescimento celular. Observou-se, à CCI óptima (CCI de  $1 \times 10^6$  células/ml), que a quantidade de células na fase S do ciclo celular é máxima e diminui com o aumento da densidade celular. Além disto, ao infectar células à mesma concentração com diferentes percentagens na fase S do ciclo celular, registou-se um aumento na productividade celular específica com o aumento da percentagem de células na mesma fase, sugerindo que a productividade celular específica é dependente do ciclo celular na altura da infecção. Para clarificar esta questão, na Parte 2, as células 293 foram sincronizadas em cada uma das fases do ciclo celular

com compostos químicos e infectadas à mesma CCI. A maior produtividade celular específica foi obtida quando as células foram sincronizadas na fase S do ciclo celular, o que confirma a nossa hipótese anterior. Assim, uma vez que o estado fisiológico das células é um parâmetro importante a considerar na altura da infecção na produção de AdV, foi desenvolvida uma estratégia para sincronizar células, sem recorrer ao uso de inibidores químicos, usando alterações da temperatura. Com esta estratégia foi possível obter uma sincronização de 57% das células na fase S do ciclo celular apenas descendo a temperatura para 31°C durante 67h e aumentando-a novamente para 37°C durante 72h. Ao infectar estas células sincronizadas obteve-se um aumento de 7.3 vezes na produtividade celular específica. Mas, devido a perdas na viabilidade celular e aos elevados tempos de incubação necessários para obter sincronização na fase S, são necessários mais estudos para “afinar” o processo.

Em anexo são mostrados dois estudos adicionais. No Anexo A, é avaliada a importância de cinéticas de infecção na optimização do processo de produção de AdV, tais como a MOI (Multiplicidade de Infecção) e HPI (Horas Pós Infecção). Em adição, o uso de meios suplementados com soro foi comparado com um meio livre de soro em termos de crescimento celular e produção de AdV; no Anexo B, é avaliado o efeito da adição de vitaminas ou lipídios na altura da infecção a elevadas densidades celulares, sem se ter verificado um aumento relevante em termos de produção de AdV.

Esta tese apresenta contribuições relevantes para a actual tecnologia de produção de AdV. Tanto a limitação do meio (quer por falta de nutrientes quer por produção excessiva de bioproductos, nomeadamente a amónia) e o estado fisiológico das células no momento da infecção foram identificados como parâmetros valiosos a ser considerados na produção de AdV a elevadas densidades celulares.



## Thesis Publications

### Journals

- Ferreira T.B., Alves P.M., Aunins J.G., Carrondo M.J.T. (2005) "Use of adenoviral vectors as veterinary vaccines", *Gene Therapy*, 12:S73-S83.
- Ferreira T.B., Ferreira A.L., Carrondo M.J.T., Alves P.M. (2005) "Two different serum-free media and osmolality effect upon human 293 cell growth and adenovirus production", *Biotechnology Letters*, 27:1809-1813.
- Ferreira T.B., Ferreira A.L., Carrondo M.J.T., Alves P.M. (2005) "Effect of refeed strategies and non-ammonogenic medium on adenovirus production at high cell densities", *Journal of Biotechnology*, 119:272-280.
- Ferreira T.B., Carrondo M.J.T., Alves P.M. (2007) "Effect of ammonia production on intracellular pH: consequent effect on adenovirus vector production", *Journal of Biotechnology*, 129:433-438.
- Zhang C., Ferreira T.B., Cruz P.E., Alves P.M., Haury M., Carrondo M.J.T. (2006) "The importance of 293 cell cycle phase on adenovirus vector production", *Enzyme and Microbial Technology*, 39:1328-1332.
- Ferreira T.B., Perdigão R., Silva A.C., Zhang C., Aunins J.G., Carrondo M.J.T., Alves P.M., "293 cell cycle synchronisation in adenovirus vector production", *Biotechnology and Bioengineering* (submitted).

### Extended Conference Proceedings

- Ferreira, T.B., Alves, P.M., Gonçalves, D., Carrondo, M.J.T. (2005) "Effect of MOI and medium composition on adenovirus infection

kinetics", Animal Cell Technology Meets Genomics, Proceedings of the 18th ESACT Meeting, Granada, Spain, May 11-14, 2003, Edited by Gòdia, F. and Fussenegger, M., Springer, 329-332.

- Ferreira T.B., Alves P.M., Carrondo M.J.T. (2007) "Effect of vitamins or lipids addition on adenovirus production at high cell densities", in Smith R (Ed), Cell Technology Processes for Cell Products, Springer (in press).

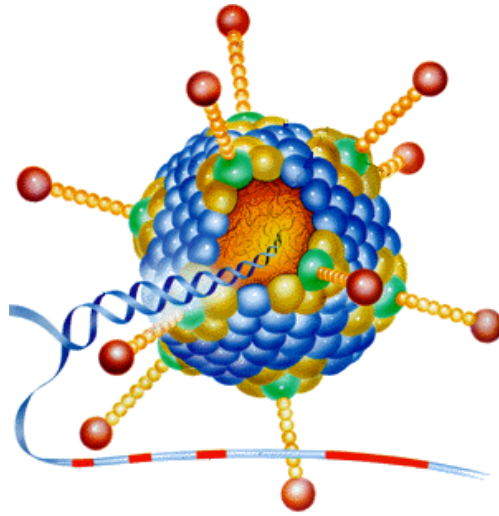
Other Publications Describing Work to Which the Author has Contributed During his Thesis Work but not Included in this Thesis

- Peixoto C., Ferreira T.B., Carrondo M.J.T., Cruz P.E., Alves P.M. (2006) "Purification of adenoviral vectors using expanded bed chromatography", Journal of Virological Methods, 132:121-126.
- Matias A., Serra A.T., Malpique R., Silva A.C., Marcelino I., Ferreira T.B., Perdigão R., Alves P., Duarte C., "Antiviral Effect of Resveratrol and a Grape Natural Extract on Adenovirus Type 5 Infection", Journal of Agricultural and Food Chemistry (submitted).
- Peixoto C., Ferreira T.B., Carrondo M.J.T., Alves P.M., "New purification platform for adenovirus vectors based on membrane technology", Biotechnology and Applied Biochemistry (submitted).

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# CHAPTER I

## Introduction



#### Use of adenoviral vectors as veterinary vaccines

Ferreira T.B., Alves P.M., Aunins J.G., Carrondo M.J.T. (2005) Gene Ther., 12:S73-S83.

## Abstract

Vaccines are the most effective and inexpensive prophylactic tool in human and veterinary medicine. Ideally, vaccines should induce a lifelong protective immunity against the target pathogen while not causing clinical or pathological signs of diseases in vaccinated people or animals. However such ideal vaccines are rare in the veterinary field. Many vaccines are either of limited effectiveness or have harmful side effects. In addition, there are still severe diseases with no effective vaccines. A very important criterion for an ideal vaccine in veterinary medicine is low cost; this is especially important in developing countries and even more so for poultry vaccination, where vaccines must sell for a few cents a dose.

Traditional approaches include inactivated vaccines, attenuated live vaccines and subunit vaccines. Recently, genetic engineering has been applied to design new, improved vaccines. Adenovirus vectors are highly efficient for gene transfer in a broad spectrum of cell types and species. Moreover, adenoviruses often induce humoral, mucosal and cellular immune responses to antigens encoded by the inserted foreign genes. Thus, adenoviruses have become singular vectors of choice for delivery and expression of foreign proteins for vaccination. Consequently, the market requirements for adenovirus vaccines are increasing, creating a need for production methodologies of concentrated vectors with warranted purity and efficacy. This chapter summarizes recent developments and approaches of adenovirus production and purification, including successes and failures in veterinary clinical applications to date. At the end of the chapter, a brief outline of the scope intended for this thesis is presented.



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## 1. ADENOVIRUS VACCINATION HISTORY

The history of veterinary vaccine development starts with the well-known story of Louis Pasteur and his rabbit spinal cord vaccine and continues to this day with the demonstration of protection in animals by rabies virus reverse transcriptase DNA plasmid vaccination (Plotkin 2000). In between, in 1947, Frenkel used suspensions of the epithelium obtained from the tongues of recently slaughtered healthy cattle that were maintained in vitro and subsequently infected in a manner similar to that used today with Baby Hamster Kidney (BHK) cells to produce Foot and Mouth Disease Virus (FMDV). The Frenkel procedure became the corner stone of vaccine production for many years and paved the way for modern biotechnology as we know it (Doel 2003). More recently, recombinant pox viruses have been generated for vaccination against heterologous pathogens, using vaccinia-vectors, expressing the rabies virus glycoprotein and Newcastle disease virus fusion and hemagglutinin glycoproteins, the first applications of genetically engineered vaccines (Paoletti 1996).

Traditional vaccination involves the use of inactivated, live-attenuated or subunit vaccines. Inactivated, or “killed” vaccines, consist of treated microorganisms that are unable to replicate; however they do not elicit protein production in the cytosol and hence viral antigens cannot be presented by MHC class I molecules, thus cytotoxic CD8 T cells are not generated. Live-attenuated vaccines are generally far more potent educating a greater number of relevant effector mechanisms, including cytotoxic CD8 T cells. Nevertheless, these vaccines sometimes have residual pathogenicity (Miller et al., 1993), or a pathogenic virus strain may re-emerge by a further series of mutations (Wood and Macadam 1997). Subunit vaccines would be as effective as live whole organisms, inherently safer than vaccines based on whole organisms, but they are not strongly immunogenic, being particularly difficult to obtain MHC class I specific responses (Yokoyama et al., 1997; Giese 1998; Dunham 2002). The

development of vaccines therefore remains an important goal of immunology. Moreover, there remains a need for further vaccines that can reduce the economic impact of disease in production animals.

The ideal vaccine should be 100% efficacious in preventing infection, although this is totally unrealistic. Therefore, the “realistic” vaccine should provide greater than 90% efficacy in disease prevention within a few days up to a couple of weeks of a single administration. Furthermore, in order to reduce the cost of regular reimmunizations and ensure receipt of a complete vaccination schedule, this protection should be of long duration, and should use a minimal number of doses. Additionally, an “ideal” vaccine would also stimulate mucosal immunity, since the majority of viruses enter via mucosal surfaces; thus, the “perfect” vaccine should be designed to be delivered by mucosal routes, i.e., intranasal or oral delivery is preferable. Vaccines also need to be safe and not cause any adverse side reaction, such as immunosuppression or interference with immunity to other vaccines given simultaneously. Finally, the vaccines need to be both genetically and thermally stable. Genetic stability must be present to ensure the absence of reversion of the live vaccine to the virulent organism which might cause disease; and thermal stability is critical since the maintenance of the cold chain is not always guaranteed from manufacturing to delivery. A very important desired characteristic is to have vaccines which can be delivered to animals at a very young age and stimulate immunity in the presence of innate, passive immunity (Babiuk et al., 1996; Jalava et al., 2002). Another very important criterion for an ideal vaccine in veterinary medicine is low cost; this is especially important in developing countries and for poultry vaccines, in which the vaccines must sell for a few cents a dose. Unfortunately, such ideal vaccines are rare in the veterinary field; additionally, there are still severe diseases with no effective vaccines.

The history of heterologous gene expression in adenovirus (Ad) goes back to the discovery of simian virus 40 (SV40) contamination in the 1950s of

inactivated Ad strains 1-5 and 7 vaccines during adaptation to growth in rhesus monkey kidney cells; it was observed that the SV40 T-antigen occasionally incorporated into the Ad genome which led to the realization that Ad could be used to express heterologous genes (Lewis 1998). Then recognition that purified replication-defective Ads could be propagated on 293 cells without helper viruses paved the way toward intentional production of genetically modified Ads (Graham et al., 1977). The popularity of Ad as a recombinant viral vector is largely due to the successful and safe immunization of millions of US military recruits in 1971 with enterically coated Ad4 and Ad7 as a prevention against acute respiratory disease (ARD) outbreaks (Bloom and Lambert 2003). Following these first trials, a number of recombinant Ad (rAd) have recently been constructed and tested not only for humans but also for veterinary vaccination (Randrianarison-Jewtougoff and Perricaudet 1995; Yokoyama et al., 1997).

Ads are highly efficacious vaccine carriers with strong immunogenicity. Although the ability of Ad vectors (AdV) to elicit antigen-specific CD8 and CD4 T cells is well described, little is known about the kinetics or nature of the immune response following Ad immunization (Yang et al., 1996; Jooss et al., 1998). However, Yang et al. (2003) observed that AdV have i) the ability to deliver large amounts of antigen into the lymphoid tissues, ii) the ability to induce rapid expansion and migration of CD8 T cells throughout the lymphatic system, and iii) the ability to produce a sustained, high-level CD8 T cell response, that may explain the strong immunogenicity of these vectors.

The use of rAd as a veterinary vaccine has many advantages: i) they can infect a wide variety of dividing or non dividing cells; ii) Ad infections are ubiquitous and are normally without significant or severe clinical symptoms (Russell 2000); iii) they can be administered orally (Babiuk and Tikoo 2000); iv) Ads have their genome well characterised (Imler 1995; Randrianarison-Jewtougoff and Perricaudet 1995); v) they can accommodate up to 8 kb of

foreign genetic material (Bett et al., 1993); vi) their genome rarely integrates into the host chromosome (Harui et al., 1999); vii) techniques are well established for the construction of AdV (Danthinne and Imperiale 2000); and viii) they have the ability to replicate at high titers in complementing cell lines (Kamen and Henry 2004).

## 2. ADENOVIRUS BIOLOGY

Ad is a non-enveloped, icosahedral virus of 60 to 90 nm with a linear duplex DNA genome of about 36 kb. The genome is divided into early (E) and late (L) genes, expressed, respectively, before and after replication of the viral chromosome (Figure 1.1). E1 gene products are involved in the control of viral gene transcription, shut-off of cellular proteins and cellular transformation. The E2 gene codes for proteins involved in viral replication, including a DNA binding protein involved in DNA elongation (E2A) and a DNA polymerase (E2B); E3 gene, dispensable for Ad replication, codes for proteins that interfere with the host immune response against virus infection; finally, the E4 genes are involved in the transition from early to late gene expression, the shut-off of host-cell gene expression, the viral replication and the assembly of the virion, (see Russell (2000) and Imler (1995) for reviews). First-generation vectors are usually deleted in their E1 and E3 regions; however, when production is performed on 293 cells, recombination between the left terminus of first-generation AdV and partially overlapping E1 sequences in the cellular genome may result in the generation of E1-positive, replication-competent Ad (RCA) (Murakami et al., 2002), a serious safety concern if non-replicating vectors are desired. Second-generation vectors are replicative-defective AdV, which are further deleted in E2A, E2B or E4, showing reduced immunogenicity and RCA generation, but engineering of stable cell lines that complement these vectors can be cumbersome and lead to poor cell growth and viral titers (Volpers and Kochanek 2004).

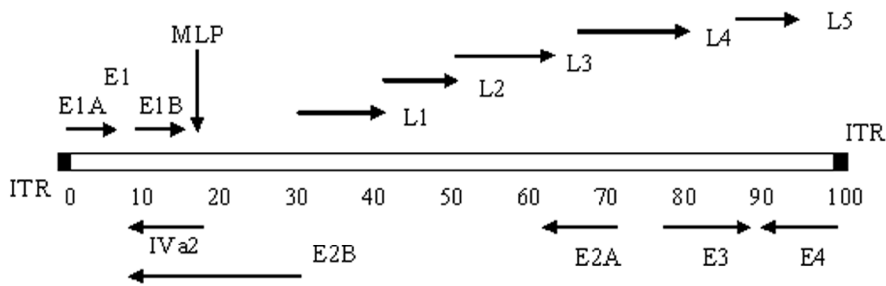


Figure 1.1. Schematic representation of the adenoviral genome organization

AdV have been developed either as replication-competent, with the expression cassette for the foreign antigen in place of the E3 genes, or as replication-defective viruses, when the antigen expression cassette replaces the E1 genes (Imler 1995). Despite the fact that it would be preferable to work with replication-defective virus, different studies have supported the use of replication-competent AdV in veterinary vaccination, since the ability of the replication-defective form to induce mucosal immunity and to protect against a respiratory challenge, following immunization, was reported to be limited (Papp et al., 1997; Fischer et al., 2002). Moreover, replication-competent AdV based vaccines were known to have the potentiality to over-ride maternal derived immunity (Papp et al., 1999; Fischer et al., 2002). Additionally, cattle vaccination with both AdVs against herpesvirus-1 showed that the use of the replication-defective form induced lower titers of antibody than the replication-competent form in intratracheal/subcutaneous immunization (Reddy et al., 2000).

### 3. SPECIES-SPECIFIC PLATFORMS

Currently, human AdV represents one of the most efficient vector systems for delivery of vaccine antigens. However, the use of human Ad (HAd) as a vaccine delivery system in non-human animals is still limited. Since non-HAd are species specific, the development of animal specific Ad as a

vaccine delivery system would be a logical choice. Thus, Ads isolated from species other than man have generated increasing interest, in particular:

i) canine Ad (CAd), that has the ability to replicate and persist locally in the upper respiratory tract of puppies in the face of maternal derived antibodies when administered via the intranasal route and use both dependent and independent coxsackievirus group B and Ad receptor (CAR) pathways to enter the cells (Soudais et al., 2000; Fischer et al., 2002);

ii) avian Ad, the subject of numerous investigations, has a number of features that make them attractive to the poultry industry: ease of propagation, ease of administration (water, aerosol or injection) and a large range of serotypes that vary in virulence. Chicken embryo lethal orphan (CELO), classified as the type 1 fowl Ad (FAd), is the most studied vector. This virus, like other typical avian Ad, has a much larger genome than mammalian Ad. Despite its large scatter, it has never been associated with serious disease or economical losses in the poultry industry. It can be isolated from healthy chickens and does not induce clinical signs when experimentally inoculated in chickens. Also this virus use both dependent and independent CAR pathways to enter the cells (Sheppard et al., 1998; Soudais et al., 2000);

iii) porcine Ad (PAd), that, depending upon serotype, can be grown in pig kidney, testis, retina, thyroid cells, human kidney, canine melanoma and calf kidney cells, do not require adjuvants for the induction of proper immune responses. Infection is generally sub-clinical causing no adverse effects in producing pigs, with PAd also isolated from apparently healthy pigs. PAd is species specific having only been isolated from swine, reducing the possibility of its spread to other animal or man following administration. PAd could induce both systemic and mucosal antibody responses (Hammond et al., 2001a; Zakhartchouk et al., 2003);

iv) and bovine Ad serotype 3 (BAd3), that, like PAd, do not require adjuvants for the induction of proper immune response, and has been used due to its lack of virulence. Moreover, experimental infections of calves

with BAd3 failed to produce either clinical signs or gross lesions, but all animals seroconverted. The cell entry pathway of BAd3 is not well understood (Benko and Harrach 1998; Reddy et al., 2000).

#### 4. APPLICATIONS

##### 4.1. Adenovirus Vaccination in companion animals

##### 4.1.1. Feline Immunodeficiency Virus (FIV)

The infection of cats with FIV results in an immunosuppressive disease that is transmitted by blood and saliva. FIV invades and destroys the monocyte/macrophage system and infects B-cells (Uhl et al., 2002). Different approaches have been made trying to immunize cats against FIV; so far, no effective vaccine has been made. Gonin et al., (1995), 10 years ago, constructed a replication-defective HAd5, containing the envelope protein ENV gene of FIV; however, despite the fact that an antibody response to pseudorabies virus in cats showed the potential of rHAd5 vectors to be used in this species, cats injected with  $10^{10.8}$ - $10^{11.8}$  of 50 percent tissue culture infectious dose (TCID<sub>50</sub>) adjuvanted with montanide ISA 708 (water in non mineral oil) or with montanide ISA 206 (double water/mineral oil/water) of this AdV did not develop detectable antibody response against ENV. Moreover, it was observed that even if high titers of antibodies against ENV products are induced, they could still be insufficient for protection (Gonin et al., 1995). Since then, no further work has apparently been performed concerning the use of AdV as a potential vaccine against FIV.



#### 4.1.2. Canine Distemper Virus (CDV)

CDV induces fatal diseases including encephalitis with demyelination, diarrhea and respiratory disorders in dogs. Although conventional live modified vaccines are commercially available and widely used in the field, their efficacy is limited in the presence of maternally-derived antibodies (Barrett 1999). Thus, a new and improved CDV vaccine which could overcome this limitation would constitute a significant improvement. The construction and characterization of the first replication-competent rCA2 started recently. The genes that code for CDV hemagglutinin (HA) (Fischer et al., 2002; Hiram et al., 2003) or fusion (F) (Fischer et al., 2002) proteins were inserted in two CA2s and used as a candidate vaccine in puppies. It was reported that intranasal vaccination with a mixture containing  $10^{5.8}$  TCID<sub>50</sub> of which rCA2 provided an excellent level of protection in seronegative puppies, inducing almost complete protection. In contrast, intranasal immunization of puppies born to CDV and CA2 immune dams failed to activate specific and protective immune responses. However, when the same puppies were vaccinated subcutaneously, significant seroconversion and solid protective immunity were triggered. Furthermore, a significant priming of memory responses was evidenced immediately after challenge, this constituting an efficient strategy to overcome both passive and active Ad specific immunity in the dog.

#### 4.1.3. Rabies Virus (RV)

Rabies still presents a health threat not only to humans but also to dogs and cats, being the dog the only important vector for humans, especially in less developed nations where uncontrolled canine rabies often is endemic. A replication-competent and -defective HA5 expressing a rabies glycoprotein (RG) has been developed, inducing immunity to rabies in rodent, canine, foxes and skunk when given by intramuscular,

subcutaneous or intranasal routes with a dose of  $10^8$  TCID<sub>50</sub>/animal. However, oral immunization failed to induce a measurable antibody response to RV using the same dose (Xiang et al., 1996; Vos et al., 2001). Dogs previously vaccinated with commercially available vaccines, immunized with  $10^7$  plaque forming unit (pfu)/animal of a replication-defective HAd5 expressing the RG, have developed higher titers of viral neutralizing antibodies against RV 10 days after vaccination when compared with conventional vaccines under similar conditions. Moreover, the immunization of dogs with the commercial non Ad vaccines is required yearly or, at best, every 3 years, depending on the type of vaccine. On the other hand, the higher antibody titers obtained with Ad vaccines against RV would reduce the frequency of dog immunization, reducing the costs for pet owners (Tims et al., 2000). One important advantage of this recombinant vaccine is the fact that the immune response to the RG was shown not to be impaired by maternal immunity; thus, this Ad vaccine is highly suitable for neonatal immunization (Wang et al., 1997).

## 4.2. Adenovirus Vaccination in Poultry

### 4.2.1. Avian Infectious Bronchitis Virus (IBV)

IBV is a highly contagious pathogen of poultry causing significant morbidity and mortality. Depending on the strain, IBV can target the respiratory tract, kidney and oviducts and result in nephritis and reduced egg production. In addition, more than 20 IBV serotypes have been identified worldwide and new serotypic variants have been identified as a result of the widespread use of live attenuated vaccines. Different approaches have been developed in order to generate a more efficacious vaccine against IBV. The expression of S1, a glycoprotein involved in the attachment of cellular receptors, by a vaccinia virus was able to induce virus-neutralizing antibodies to IBV when delivered to mice; however, multiple injections are

required to achieve a reasonable degree of protection. Recently, a FAd expressing the S1 of IBV has been developed (Johnson et al., 2003). A single dose of  $10^6$  TCID<sub>50</sub>/animal was shown to be sufficient to obtain complete protection of chickens at the trachea, the primary site of infection by IBV. Moreover, even in the face of FAV maternal antibodies, a high level of protection was achieved (Johnson et al., 2003).

#### 4.2.2. Infectious Bursal Disease Virus (IBDV)

IBDV induces an immunosuppressive disease of chickens by destruction of the B-lymphocytes. Current vaccination alternatives consist of either live virus or inactivated oil-emulsion vaccines, which induce serum antibody production in breeding hens after natural exposure; they are transferred to the progeny chicks via the yolk sac providing protection for the first critical weeks after hatching (Muller et al., 2003). The construction of a rFAd10 containing the VP2 gene from IBDV has been described (Sheppard et al., 1998). This recombinant vaccine was shown to induce an immune response in chickens to VP2 after vaccination with  $10^7$  pfu/animal. Moreover, after challenge with IBDV, intravenously, intraperitoneally, subcutaneously or intramuscularly vaccinated chickens were protected, although no protection was observed in conjunctival sac vaccinated chickens (Sheppard et al., 1998).

#### 4.3. Adenovirus Vaccination in Swine

##### 4.3.1. Classical Swine Fever Virus (CSFV)

Classical swine fever (CSF), also known as hog cholera, is a serious and contagious viral disease of pigs with a high mortality rate, difficult to control in areas of high pig or wild boar densities, being the most economically important disease of swine in areas of intensive pig farming.

For this reason, CSF is included in the A list of infectious diseases of the highest importance for international trade. Thus, it is highly relevant to develop efficacious vaccines for the control of CSFV in domestic pigs and in wild boar. Prophylactic vaccination is still carried out in many parts of the world (Paton and Greiser-Wilke 2003). New vaccine developments have included a number of different strategies for delivering the major envelope glycoprotein, E2, against which most neutralizing antibodies are directed (van Oirschot 2003). Hammond et al. (2000), constructed the first rPAd expressing the E2 protein and showed that a single dose of  $10^7$  TCID<sub>50</sub>/animal in tissue culture supernatant, when administered subcutaneously, is sufficient to completely protect pigs against subcutaneously challenge. Later, the same authors showed that when the challenge is administered orally, only 60% of the animals were protected (Hammond et al., 2001b); more, recently, it was shown that pigs given two oral doses of  $10^6$  TCID<sub>50</sub>/animal of rPAd were completely protected from the disease (Hammond et al., 2003).

#### 4.3.2. Pseudorabies Virus (PrV)

PrV is an alpha herpesvirus which causes the economically important and widespread Aujeszky's disease (AJD) in pigs. PrV is a highly neurotropic virus causing nervous and respiratory complications in pigs, the natural host, and in a variety of other animal species. Vaccination against AJD is widely practised with live attenuated or killed whole virus vaccines. However, neonatal immunization is often limited in the presence of maternally-derived antibody, which inhibits the immune response against both vaccines (Roth 1999). Recently, the use of rAd vaccines carrying individual PrV genes were constructed as a safe alternative. Glycoproteins gD, gB and gC of PrV were chosen on the basis of their role in eliciting a protective immune response against virus infection. It was shown that piglets vaccinated intramuscularly with  $10^{8.6}$ - $10^{9.6}$  TCID<sub>50</sub>/animal one day

after birth, with replication-competent HAd5 harbouring these three genes, developed similar neutralizing antibody responses independently of the presence or absence of maternal antibodies and were partially protected against challenge 16 weeks later (Monteil et al., 2000). For pigs that are slaughtered a few months after birth, one-shot vaccination at birth could provide protection of sufficient duration. Finally, two doses of 2 ml of clarified tissue culture supernatant containing  $10^{5.4}$  TCID<sub>50</sub> of rPAd expressing the PrV gD gene were administered subcutaneously and showed to protect pigs after challenge. Post-mortem, gross lesions of pneumonia were found in the lungs of pigs given a single dose of vaccine; the lungs of pigs given two doses were free from disease (Hammond et al., 2001a).

#### 4.3.3. Foot and Mouth Disease Virus (FMDV)

Foot and mouth disease (FMD) is a severe, clinically acute, vesicular disease of cloven-hoofed animals including domesticated ruminants, pigs and more than 70 wildlife species. Pigs are recognized as a significant factor in the spread of the disease since a single pig releases as much aerosol virus as 3000 cattle in a short period of time (Alexandersen et al., 2003; Thomson et al., 2003). The economic and social impact of FMD can be catastrophic when an outbreak occurs in FMD-free countries populated with immunologically naive animals. The current vaccine is a chemically inactivated preparation of concentrated infected cell culture supernatant. However, FMD-free countries generally prohibit its use because of the lack of an approved diagnostic test that can reliably distinguish vaccinated from infected animals. Moreover, current vaccines can induce a protective response only after approximately 7 days post vaccination; this is a critical issue in disease-free countries, where, in the case of FMD outbreaks, a rapid control in preventing the spread of the disease is crucial (see Doel (2003) for a review). Thus, there is a need to develop disease control strategies relying on more rapidly induced protection. Mayr et al. (1999)

developed a replication-defective HAd5 vector containing the capsid polypeptide P1 and the viral 3C protease coding regions, necessary for processing P1 to the capsid proteins VP0, VP3 and VP1, from the FMDV strain A12. They observed that vaccinated swine with  $1 \times 10^8$  pfu/animal in PBS developed antibodies against FMDV structural proteins and an FMDV-specific neutralizing antibody response which seems to increase slightly by boosting the swine with a second inoculation of  $5 \times 10^8$  pfu/animal in PBS at 4 weeks post-initial vaccination (Mayr et al., 2001). This vaccination protocol was shown to offer a significant degree of protection to the pigs, as five of six pigs were completely protected, while the remaining animal had significantly reduced signs of disease. Later, Moraes et al. (2002) showed that a single dose of  $5 \times 10^9$  pfu/animal in PBS of a replication-defective HAd5 expressing the P1 coding region of FMDV strain A24 completely protected pigs against homologous challenge 7, 14 or 42 days after vaccination. Since efficacious vaccination is strain dependent and the infection with one serotype does not confer protection against another, HAd5 bicistronic vector vaccines have been developed (Wu et al., 2003), decreasing the cost for multivalent adenoviral FMD vaccines. For the construction of these vectors, the P1 capsid coding region for both A24 and O1 strains and the 3C protease coding region of A12 strain were used. However, the neutralizing antibody response after vaccination with  $2.5 \times 10^9$  pfu/animal in PBS of the bicistronic vector was considerably lower than that induced by a commercial FMD vaccine or the monovalent Ad-A24 vaccine (Moraes et al., 2002). Recently, a new strategy based on the fact that FMDV is highly sensitive to alpha/beta interferon (IFN- $\alpha/\beta$ ) have been developed using a replication-defective HAd5 (Chinsangaram et al., 2003; Moraes et al., 2003). This strategy has been shown to completely protect pigs after vaccination with  $10^9$  pfu/animal when challenged 24 h later with virulent FMDV.

#### 4.3.4. Porcine Respiratory and Reproductive Syndrome Virus (PRRSV)

PRRSV is the causative agent of an economically important pig disease, with a worldwide distribution, characterized by reproductive failure in sows and respiratory problems in unweaned and growing pigs. Swine macrophage is the only cell type known to support PRRSV replication, making commercial production impossible. Direct contact between infected and naive pigs is the predominant route of PRRSV transmission. Moreover, pneumonia caused by PRRSV infection is more severe in young pigs compared to adults and may be complicated by concurrent bacterial infection (Rossow 1998). Gonin et al. (1999) observed that PRRSV infected pigs present circulating antibodies responsible for viral neutralization mainly directed against GP<sub>5</sub>, an envelope protein. Gagnon et al. (2003) constructed a replication-defective HAd5 expressing the GP<sub>5</sub> protein and used this recombinant virus to immunize pigs using two intradermal injections with  $5 \times 10^8$ - $1 \times 10^9$  pfu/animal in a mixture containing 100  $\mu$ L of PBS and 100  $\mu$ L of poloxamer SP1017 at 0.02%. It was observed that following challenge given intranasally 14 days after the booster, pigs produced high antibody titers to GP<sub>5</sub> protein. Moreover, vaccinated pigs presented specific immune memory which, following a subsequent PRRSV infection, resulted in a rapid clonal expansion of memory cells to the neutralizing epitopes of the authentic viral GP<sub>5</sub> protein.

#### 4.3.5. Transmissible Gastroenteritis (Corona) Virus (TGEV)

TGEV infects the enteric and respiratory tissues of newborn piglets resulting in mortalities approaching 100%. The virus infects epithelial cells and, in some cases, lung macrophages (Garwes 1988). There are several commercially available TGEV vaccines, both inactivated and attenuated; these do not fully protect piglets. Several attempts have been made to

develop efficacious recombinant TGEV vaccines (Tuboly and Nagy 2001). The spike protein (S) was identified as the major inducer of TGEV-neutralizing antibodies and it mediates binding of TGEV to its cellular receptor (Jimenez et al., 1986). Thus, HAd5 expressing the S protein was constructed and used to study the induction of antibodies providing protection in swine (Torres et al., 1995). It was observed that porcine serum, elicited by  $10^9$  pfu/animal in PBS of this recombinant, when mixed with a lethal dose of virus prior to administration to susceptible pigs, prevented the replication of virulent TGEV administered orally as virus-antibody mixtures and fully protected swine from clinical signs and death. Moreover, the used dose did not produce any clinical symptoms in any of the more than 50 animals inoculated up to 10 weeks after inoculation (Torres et al., 1996), suggesting that this vector can be used as a live vaccine in swine without secondary complications associated with the vector. However, a PAd vector could be more effective than HAd. Thus, Tuboly and Nagy (2001) constructed a rPAd5 expressing the TGEV protein. It was observed that a single oral dose of  $5 \times 10^6$  pfu/animal of the recombinant virus was sufficient to induce both a systemic and a local humoral immune response (Tuboly and Nagy 2001). Unfortunately challenge experiments were not carried out.

#### 4.3.6. Swine Influenza Virus (SIV)

SIV is a widespread and important pathogen in species as diverse as poultry, swine, marine mammals and humans. In pigs, influenza can occur either as an enzootic problem in a herd or, more commonly, as explosive outbreaks of acute respiratory disease. Although rarely fatal, swine influenza can be of substantial economic impact (van Reeth and Nauwynck 2000). In addition, there is growing concern for the potential for synergistic infections with influenza and PRRSV. Beyond the impact of influenza for the swine industry, pigs are also very important in the global ecology of



influenza A viruses in humans (Zhou et al., 1999). SIV vaccines that are commercially-available are inactivated, whole-virus or subunit vaccines. While these vaccines may decrease the incidence and severity of clinical disease, they do not consistently provide complete protection from virus infection. Two replication-defective HAd5 were developed as potential vaccines against SIV: rHAd5 expressing the influenza virus H3 haemagglutinin (HA) (Tang et al., 2002; Wesley et al., 2004) inducing predominately a subtype-specific humoral immune response (Macklin et al., 1998); and rHAd5 expressing the nucleoprotein (NP) (Wesley et al., 2004), a group-specific stimulating cytotoxic T lymphocytes for cross-reactive immunity to all influenza A subtypes (Ulmer et al., 1998). It was observed that the immunization of mice with  $5 \times 10^8$  TCID<sub>50</sub>/animal and pigs with  $2 \times 10^{10}$  TCID<sub>50</sub>/animal in PBS with the first recombinant, described above, developed high levels of virus-specific hemagglutination-inhibition antibody to SIV by 4 weeks post vaccination and the animals were partially protected. On the other hand, pigs vaccinated with  $2 \times 10^{10}$  TCID<sub>50</sub>/ml in PBS of both recombinant viruses in a mixture were completely protected.

#### 4.4. Adenovirus Vaccination in Cattle

##### 4.4.1. Bovine Viral Diarrhea Virus (BVDV)

BVDV is responsible for reduced milk production, reduced reproductive performance and growth retardation. In addition, acute infection in adult cattle, congenital defects and increased neonatal mortality are also clinical manifestations of BVDV infection (Goens 2002). Finally, it was reported that BVDV may play an indirect role in immunosuppression (Wellenberg et al., 2002). Currently, inactivated and modified-live vaccines are used; however, both types of vaccines have significant shortcomings. A rHAd5 expressing the nucleocapsid C protein (p14), that is highly conserved among many different pestiviruses, to which BVDV belongs, was

constructed and shown to induced both humoral and cellular immune responses after vaccination with  $10^9$  pfu/animal, in a mouse model (Elahi et al., 1999a). The E2 protein of BVDV, which is a major viral glycoprotein, was also used for mice immunization using the same vector and dose (Elahi et al., 1999b). A strong humoral immune response was detected as the presence of a strong memory response to the E2 protein. Both strategies used an inducer/promoter allowing the generation of rAd in which the production of the transgene may be toxic and permitting the control of the transgene expression in vitro and in vivo. Finally, a rBAd3 expressing BVDV E2 protein was constructed and shown to induce BVDV E2-specific mucosal and systemic immune responses after intranasal immunization with  $10^7$  pfu/animal of cotton rats (Baxi et al., 2000). Despite the fact that mice immunized with recombinant fowl pox virus or with a DNA vaccine expressing E2 glycoprotein induced E2-specific immune responses, it was also observed that the neutralization titers were low (Baxi et al., 2000).

#### 4.4.2. Bovine Parainfluenza Virus Type 3 (bPIV3)

bPIV3 has been isolated from normal cattle, cattle showing signs of respiratory disease and aborted fetuses. An intramammary inoculation of bPIV3 resulted in respiratory signs, fever and malaise. Moreover, the milk showed a color change, an increased pH and increased numbers of glandular epithelial cells, neutrophils, lymphocytes and monocytes (Wellenberg et al., 2002). Currently, multivalent vaccines containing killed bPIV3 are used. Breker-Klassen et al. (1995) constructed two rHAd5 encoding either the F or the HN protein of bPIV3. It was shown that intranasal vaccination with  $5 \times 10^7$  pfu/animal in PBS of cotton rats produced a strong serological response within 21 days which increased with the second vaccination. Moreover, F and HN proteins produced by rHAd5 either alone or in combination were sufficient to induce a protective immune response (Breker-Klassen et al., 1995).

#### 4.4.3. Bovine Herpes Virus 1 (BHV-1)

BHV-1 causes a variety of diseases in cattle including infectious bovine rhinotrachitis, infectious pustular vulvo-vaginitis, conjunctivitis and occasionally meningo-encephalitis. Live attenuated and killed vaccines are partially effective in reducing the disease incidence and severity (Wellenberg et al., 2002). The limitations with the existing vaccines have encouraged the development of alternative, cost effective vaccination strategies such as recombinant live viral vector based vaccines. Mittal et al. (1996) developed the first replication-competent and -defective HAd5 carrying the gD glycoprotein gene from the BHV-1 envelope. They showed that both forms induced immunity in cotton rats after vaccination with  $10^7$  pfu/animal in PBS and no infectious BHV-1 virions were isolated from the trachea of these rats after challenge. The same strategy was used with a rBAd3 and tested in its ability to induce mucosal and systemic immune response in cotton rats (Zakhartchouk et al., 1998) and calves (Reddy et al., 2000) with success. More recently, newborn lambs were immunized with  $2 \times 10^9$  pfu/animal of replication-defective HAd5 expressing the gD protein of BHV-1 (Mutwiri et al., 2000). Both humoral and cell-mediated gD-specific mucosal immune responses were detected in 1 to 4 days old lambs after a single immunization and these responses were qualitatively and quantitatively similar to those detected in 5 to 6 weeks old lambs. Moreover, gD-specific maternal antibody did not significantly alter either mucosal or systemic gD-specific immune response. In addition, BHV-1 glycoprotein gC was also tested in its ability to induce protective immunity to BHV-1 in cattle by intranasal administration of  $10^{10}$  pfu/animal using a replication-defective HAd5 (Gogev et al., 2002). However, it was observed that the highest BHV-1 neutralizing antibody titers were obtained with rHAd5 expressing the gD protein followed by the live vaccine.

#### 4.4.4. Rinderpest Virus (RPV) and Peste des Petits Ruminants Virus (PPRV)

Because of their high mortality and high morbidity rates, RP and PPR are dreaded animal diseases which are drawing back the animal production in many developing countries (Africa and Asia). They affect both domestic and wild ruminants and belong to the list A of the Office International des Epizooties where highly contagious animal diseases with high economic importance are grouped. Currently, attenuated tissue culture vaccines used to control RP and PPR viruses have been successfully used for many years. Safe and effective they provide complete and lifelong protection with a single subcutaneous inoculation (Yamanouchi and Barrett 1994; Diallo 2003). However, for vaccination programs, the oral route of administration would be more economical. Moreover, the parenteral way of administration means that the current vaccine cannot be used in wildlife. A production, purification, and encapsulation processes for a replication-defective HAd5 expressing two different morbillivirus capsids proteins, the responsible agent of both diseases, is in progress. The aim is the development of heat stable RP/PPR oral vaccines better adapted for mass vaccination in hot climate developing countries. Animal vaccination and challenge testes are ongoing with promising preliminary results.

In summary, according to the numerous reported studies, live human or non human rAds, either infectious or not, appear to be attractive candidates for vaccination against several animal disease (Table 1.1). Success situations are clear against most pathogens reported and intermediate successes are apparent against CDV and RPV/PPRV, whereas vaccination against FIV have, thus far, yielded poor or inconclusive results. Nevertheless, this is not a comprehensive picture, since more than 50 viral agents of veterinary importance are known (Table 1.2).

Table 1.1. Host and pathogens for which Ad vaccines have been tested in companion animal, poultry and livestock.

Species	Pathogen	Protection	Reference(s)
Feline	FIV	No	(Gonin et al., 1995)
Canine	CDV	Partial	(Fischer et al., 2002; Hirama et al., 2003)
	RV	Yes <sup>1</sup>	(Xiang et al., 1996; Wang et al., 1997; Tims et al., 2000; Vos et al., 2001)
Chicken	IBV	Yes	(Johnson et al., 2003)
	IBDV	Yes	(Sheppard et al., 1998)
Swine	CSFV	Yes	(Hammond et al., 2000; Hammond et al., 2001b; Hammond et al., 2003)
	PrV	Yes	(Monteil et al., 2000; Hammond et al., 2001a)
	FMDV	Yes	(Mayr et al., 1999; Mayr et al., 2001; Moraes et al., 2002; Chinsangaram et al., 2003; Moraes et al., 2003; Wu et al., 2003)
	PRRSV	Yes	(Gagnon et al., 2003)
	TGEV	Yes	(Torres et al., 1995; Torres et al., 1996; Tuboly and Nagy 2001)
	SIV	Yes	(Macklin et al., 1998; Tang et al., 2002; Wesley et al., 2004)
Bovine	BVDV	Yes <sup>2</sup>	(Mittal et al., 1996; Elahi et al., 1999a; Baxi et al., 2000)
	bPIV3	Yes <sup>2</sup>	(Breker-Klassen et al., 1995)
	BHV-1	Yes	(Mittal et al., 1996; Zakhartchouk et al., 1998; Mutwiri et al., 2000; Reddy et al., 2000; Gogev et al., 2002)
	RPV / PPRV	Partial	Unpublished

<sup>1</sup>Obtained in mice; <sup>2</sup>Obtained in cotton rats.

Table 1.2. Over 50 viral agents available in the veterinary vaccine market

Species	Pathogen
Bovine	Coronavirus; Foot and Mouth Disease O, A, C; Infectious Rhinotracheitis; Papillomavirus; Parainfluenza 3; Respiratory Syncytial Virus; Rotavirus; Bovine Viral Diarrhea 1 and 2
Porcine	Encephalomyocarditis; Influenza; Parvovirus; Pseudorabies; Reproductive and Respiratory Syndrome; Rotavirus; Transmissible Gastroenteritis; Rabies
Equine	Eastern Encephalitis; Venezuelan Encephalitis; Western Encephalitis; Herpesvirus 1 and 4; Influenza; Rabies
Caprine/Ovine	Bluetongue; Ecthyma (Orfvirus); Rabies
Avian	Encephalomyelitis; Fowl Pox; Hemorrhagic Enteritis; Infectious Bronchitis; Infectious Bursal Disease; Laryngotracheitis; Marble Spleen Disease; Marek's Disease; Newcastle Disease; Paramyxovirus; Pigeon Pox; Reovirus; Tenosynovitis
Feline	Calicivirus; Leukaemia; Panleukopenia; Peritonitis; Rabies; Rhinotracheitis; Lentivirus
Canine	Adenovirus; Coronavirus; Distemper; Hepatitis; Measles Parainfluenza; Parvovirus; Rabies

## 5. ADENOVIRUS PRODUCTION AND PURIFICATION AS VETERINARY VACCINES

Process design is critical when developing cost effective veterinary vaccines. The goal of minimising the number of process steps is a prerequisite if industrial application is intended. In addition, veterinary vaccines do not impose the same final purity requirements as human vaccines, which should reflect on the purification schemes.

The production of Ad vaccines for veterinary applications is mainly based upon the use of continuous cell lines such as 293 (Graham et al., 1977),

MDBK (Reddy et al., 2000; Fischer et al., 2002), VR1BL, HeLa and A549 (Zakhartchouk et al., 2003). During the cell proliferation step, various animal sera are used to enhance cell growth. However, supplementing cell culture media with such components presents several drawbacks like lot to lot variation, potential risk of contamination by viruses, mycoplasma, prions, etc. (Freshney 1986). Furthermore, regulatory authorities in Europe (European Medicines Agency (EMA)) and in the United States (Food and Drug Administration (FDA)) have encouraged biological manufacturers to reduce or eliminate the use of substances of animal origin in their manufacturing processes. Recently, several serum and protein free culture media became commercially available. Although the use of such defined media is still expensive, the large scale production of AdV will require the use of this type of culture media to facilitate downstream processing and, in time, decrease the overall process cost.

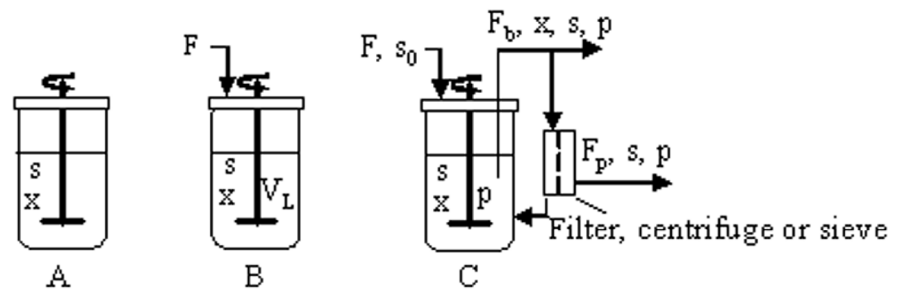


Figure 1.2. Bioreactors operation modes: A) Batch; B) Fed-batch; C) Perfusion; s = Substrate; x = Cells; F = Flow of fresh substrate; F<sub>b</sub> = Bleed, flow of broth with cells out of the bioreactor; F<sub>p</sub> = Permeate flow, cell free flow of product; V<sub>L</sub> = Working volume; p = Product; S<sub>0</sub> = Substrate in the flow.

Production methods differ according to the cell type used: adherent versus suspension cell culture. For manufacturing purposes, suspension adapted cell lines are more convenient for production at large scale and different operation modes have been developed for Ad production (Figure 1.2): i)

batch mode providing the easiest way to proceed as no extra feeding is required and the risk of contamination is lowered given the simplicity of operation; ii) fed-batch mode, easy to operate and readily scalable, is employed to extend culture lifetime by supplementing limiting nutrients or reducing the accumulation of toxic metabolites; and iii) perfusion mode, consisting in cell retention at a relatively high concentration inside the bioreactor, while fresh nutrient supply and metabolite removal takes place (see Kamen and Henry (2004) for a review).

Methodologies for production of concentrated AdV at low cost are mandatory as the market needs for Ad are increasing. Although Ad has the advantage to be produced at high titers ( $10^{10}$ - $10^{11}$  pfu/ml) (Babiuk and Tikoo 2000), to obtain a good immune response in a large proportion of treated animals, particularly for mucosal vaccination, large doses and thus culture volumes are required. Further process development aiming at higher yields of product is clearly necessary. Improvements in volumetric production can be achieved by increasing the cell density at which cells can be infected without lowering the specific yield of the product; however, production of AdV that maintain a high specific yield in batch operation is limited to cell densities in the range of  $1 \times 10^6$  cell/ml; several approaches have been made in order to overcome this so called "cell density effect" (Nadeau and Kamen 2003): Garnier et al. (1994) demonstrated that medium replacement at infection and the addition of glucose at 24 hours post infection (hpi) together with periodical pH adjustments, allowed a sustained maximum specific productivity at  $1.6 \times 10^6$  cell/ml whereas Nadeau et al. (1996), further improved this strategy by also adding essential amino acids at 24 hpi thereby stabilising volumetric productivity at cell densities above  $2 \times 10^6$  and below  $3 \times 10^6$  cell/ml. Such results hint at the existence of substrate limitation and/or byproduct inhibition at high cell densities. At production scale, medium exchange will increase the cost of the final product, which is even more critical when this is to be used in the veterinary field. Perfusion mode operation has been attempted as a



means to control the culture environment and remove toxic byproducts (Henry et al., 2004; Kamen and Henry 2004); nevertheless, the cell specific productivity could only be maintained by infecting cells at densities up to  $3 \times 10^6$  cell/ml using high perfusions rates of 2 reactor volumes per day, at 2 days post infection, a very costly proposition (Kamen and Henry 2004).

The importance of infection kinetics on Ad production and the significance of variables such as Multiplicity Of Infection (MOI) and harvesting time in process optimisation is also mandatory to increase production yields, to avoid rapid depletion of costly and certified master virus banks as well as to ensure that the infection kinetics is reproducible between different production scales. Moreover, the use of low MOI at large scale would be preferential since an intermediate step of virus inoculum production would be avoided (Annex A).

Finally, veterinary products should be purified with a minimal number of steps and the unit operations employed should be simple and non-expensive. Traditionally, laboratory purification of rAd was achieved using two rounds of cesium chloride (CsCl) density gradient ultracentrifugation. However, CsCl density gradient method is not scaleable. Despite the fact that chromatographic purification is an expensive method, ion exchange, hydrophobic interaction, metal chelate and size-exclusion chromatography have been evaluated for capture and purification of HAd5 (Huyghe et al., 1995). Kamen and Henry (2004) developed a method for gene therapy application consisting of: i) harvest of infected cells by continuous centrifugation, ii) cell lysis by osmotic shock, iii) DNase treatment with centrifugal/conditioning, iv) filtration, v) anion-exchange chromatography, vi) ultrafiltration/concentration and vii) size exclusion chromatography. This protocol allows large scale purification of AdV with purity comparable to the CsCl gradient method; however, it increases the final product price, well beyond the reach of veterinary utilisation. Introgen developed a method consisting of a single ion chromatography run, after concentration/diafiltration and nuclease treatment, with a total recovery

of the virus product of 70% (Zhang et al., 2001). Also, a protocol to purify HAd5 from the bulk harvested directly from the bioreactor after lysis (without a concentration step) was developed using a single ion exchange chromatographic step followed by ultrafiltration with a final yield of 32% achieved in less than one working day with a minimal amount of sample manipulation (Peixoto et al., 2005). This process presented the advantage of capturing the viral particles directly from cellular extracts and to account for the significant amounts (25-80%) of AdV that are present in the culture medium at harvest time due to early cell lysis (Schagen et al., 2000).

## 6. SCOPE OF THE THESIS

One of the main goals of a commercial AdV cultivation process for gene therapy or vaccine applications is to maximise the virus volumetric productivity, defined as units of viral infective particles, per culture volume per unit time. Since one can infect at different cell concentrations, the cell specific productivity, defined as viral infective particles produced per cell and unit time, is a parallel concept; ideally, operating at the highest possible cell specific productivity obtained at the highest cell concentration at infection would maximize the volumetric productivity of the process but quality of the produced AdV is of paramount importance.

This thesis has attempted to improve the current manufacturing process of AdV production by performing infection at high cell densities maintaining the cell specific productivity. To achieve this, the bottlenecks in cell productivity have been studied as well as the parameters that may affect the vector production at high cell densities. The cell density at infection is a very important parameter as it strongly impacts the AdV volumetric productivity. However, in simple batch-mode operations, a relatively narrow range for optimal cell density at infection of  $1 \times 10^6$  cell/ml has been consistently reported. The reasons for the drop in per cell productivity at

higher cell densities are not currently known, as medium formulations allow maximum cell growths reaching  $8 \times 10^6$  cell/ml. Therefore more research is needed to understand and overcome this phenomenon to enable simple batch processes to reach higher productivities. In order to design a rational production process that allow the maximisation of the AdV yields at high cell densities, four different bioreaction approaches were explored in this thesis: i) media exchange, ii) refeed strategy, iii) use of non-ammoniagenic medium and iv) cell cycle synchronisation. The knowledge gained in this scientific excursion is integrated into an improved process.

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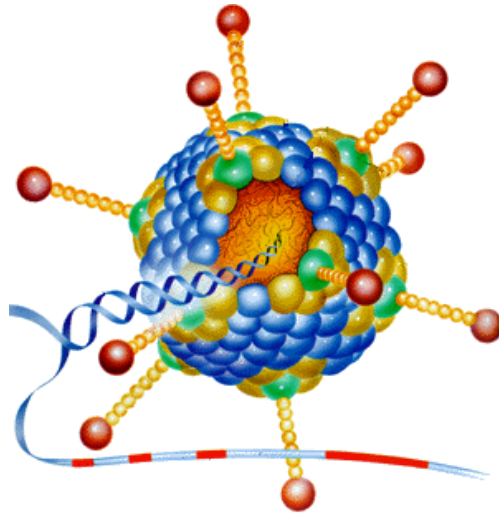
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## CHAPTER II

### Cell Density Effect





Two different serum-free media and osmolality effect upon human 293 cell growth and adenovirus production

Ferreira T.B., Ferreira A.L., Carrondo M.J.T., Alves P.M. (2005) *Biotechnol. Lett.*, 27:1809-1813.

## Abstract

Adenoviruses are promising vectors for gene therapy and vaccination protocols. Consequently, the market requirements for adenovirus are increasing, driving the search for new methodologies for large-scale production of concentrated vectors with warranted purity and efficacy, in a cost-effective way. Nevertheless, the production of adenovirus is currently limited by the so-called “cell density effect”, i.e., a drop in cell specific productivity concomitant with increased cell concentration at infection. In this study, two different serum-free culture media (CD 293 and EX-Cell) are evaluated in their effect on human 293 cells growth and adenovirus production at cell densities higher than  $1 \times 10^6$  cells/ml. The results show that EX-Cell was the better medium for cell growth. Although adenovirus production was equivalent in both CD 293 and EX-Cell media when the infection was performed at  $1 \times 10^6$  cells/ml, at  $3 \times 10^6$  cells/ml CD 293 proved to be the better medium. This result was related with the high ammonia content in EX-Cell medium at the highest cell concentration at infection. Besides this, the large-scale production of these vectors at high cell densities often requires refeed strategies, which increase medium osmolality. While a negative effect on cell growth was observed with increasing osmolalities, adenovirus productivity was only affected for osmolalities higher than 430 mOsm.

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## 1. INTRODUCTION

Recombinant adenoviruses have been the vectors of choice for gene therapy and vaccination due to their high efficiency for gene transfer in a broad spectrum of cell types (Rea et al., 1999; Mountain, 2000). Therefore, market needs for adenovirus vectors (AdV) are constantly increasing causing a high demand of methodologies for the large-scale production of concentrated vectors with warranted levels of purity and efficacy at a low cost (Ferreira et al., 2005a). Different approaches for the improvement of production and purification processes of these viral vectors have mostly used serum-free media (Nadeau et al., 1996; Cote et al., 1997; Cote et al., 1998). Serum-free media formulations are, however, more expensive than the traditional ones with serum supplementation and also the cell specific productivity decreases sharply with increased cell concentration at infection (CCI) above  $1 \times 10^6$  cell/ml, the so called “cell density effect” (Nadeau and Kamen 2003; Kamen and Henry 2004). The reasons for such an effect are not very well understood but the lack of an essential nutrient or growth factors or high concentration of metabolic inhibitory products, present at high cell densities, are the most probable causes. Recently, several commercial serum-free media have become available; thus, one of the goals of this study was to evaluate the effect of two different commercially available serum-free media on human 293 cell growth and AdV productivity at high cell densities.

Besides culture media composition and the accumulation of inhibitory byproducts, several other factors have been reported to influence cell growth and AdV productivity, namely the vector itself, the cell line, the pH, pCO<sub>2</sub> and temperature (Eloit and Adam 1995; Iyer et al., 1999; Jardon and Garnier 2003; Nadeau and Kamen 2003). Due to the common need to implement refeeding strategies for AdV production, it is pertinent to determine if the consequent increase in osmolality has an influence on cell growth and AdV productivity. Only one study has reported the influence of

osmolality on protein production by human 293 cells (Nadeau et al., 1996), resulting in increased productions at high osmolalities (500 mOsm). Nevertheless, the effect of osmolality on viral productivity has not been assessed yet, thus this study reports effect of osmolality upon human 293 cell growth and AdV productivity.

## 2. MATERIALS AND METHODS

### 2.1. Cell line and culture conditions

Suspension-adapted human 293 cells (ATCC-CRL-1573) were grown in 125 ml Erlenmeyer flasks, with 40 ml medium in two different serum free media: CD 293, a protein-free medium from Invitrogen and EX-Cell, an animal-protein free medium from JRH, all supplemented with 6 mM glutamine from Invitrogen. Cells were incubated at 37 °C in a humidified atmosphere of 8 and 10 % of CO<sub>2</sub> in air, respectively (in order to obtain an equal pH in each medium), with a cell inoculum of 0.25×10<sup>6</sup> cell/ml. Regarding the osmolality studies, five different osmolalities were evaluated: 230, 330, 380, 430 and 480 mOsm. The osmolality of the medium was increased by addition of a 1 M NaCl.

### 2.2. Infection in shake flasks

A replication-defective AdV derived from type 5 AdV was kindly provided by Dr. Tom Barret (Institute for Animal Health-Pirbright, UK). Infection was made when the cells reached a concentration of 1×10<sup>6</sup> cell/ml using a multiplicity of infection (MOI) of 10 (see annex A). Infection was also made when cells reached a concentration of 2×10<sup>6</sup> cell/ml and 3×10<sup>6</sup> cell/ml for the studies on media effect, maintaining the same MOI. Infected human 293 cells were harvested at 48 h post infection (hpi), determined as the optimal harvest time (Annex A).

### 2.3. Virus culture samples preparation

Infected human 293 cells were harvested at 48 hpi and centrifuged at 1000 g for 10 min at 4 °C; the resulting cell supernatant was distributed into small aliquots and stored at -85 °C for the evaluation of extracellular virus; the resulting cell pellet was resuspended in a known volume of lysing buffer (Tris/HCl 10 mM, pH 8.0, 2mM MgCl<sub>2</sub> and 0.1% Triton-X 100) and the cells were disrupted by 1 minute vortex. Cell debris was removed by centrifugation at 3000 g for 10 min at 4 °C and the resulting cell lysate supernatant was distributed into small aliquots and stored at -85 °C for quantification of intracellular viruses content.

### 2.4. Analytical Methods

Cell concentration and viability were determined by counting the cells on a haemocytometer using the Trypan Blue dye exclusion method.

AdV titration was performed by the end-point dilution method using 96 well plates and human 293 cells. Infectious particles (ip) in 50 % tissue culture infective dose (TCID<sub>50</sub>) were determined according to the statistical method of Spearman-Kärber (Darling et al., 1998).

Ammonia was quantified enzymatically using a UV-test Cat. No. 1112732035 (Boehringer Mannheim, R-Biopharm AG)

Osmolality was measured in a Digital Micro Osmometer Type 5R from Hermann Roebling MESSTECHNIK, Germany.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of media on cell growth

In order to obtain high cell yields for AdV production, two different serum-free media were tested in their ability to grow human 293 cells. As shown

in Figure 2.1, EX-Cell allowed a maximum cell number of  $7.5 \times 10^6$  cell/ml, while the maximum cell number obtained in CD 293 was approximately  $4 \times 10^6$  cell/ml, attained at similar specific growth rates ( $0.018 \text{ h}^{-1}$  and  $0.019 \text{ h}^{-1}$  for CD 293 and EX-Cell, respectively). Despite the fact that EX-Cell has a higher glucose concentration (30 mM) than CD 293 (25 mM), the main difference observed between the two media composition is in the amino acids concentration. By following amino acid consumption it was seen that threonine was the unique amino acid that was completely consumed when cells reached  $4 \times 10^6$  cell/ml in CD 293 (data not shown). Furthermore, EX-Cell contains a concentration of threonine 10 times higher than CD 293, and the refeeding of this amino acid in CD 293 led to an increase in maximum viable cell concentration to  $5.5 \times 10^6$  cell/ml (data not shown).

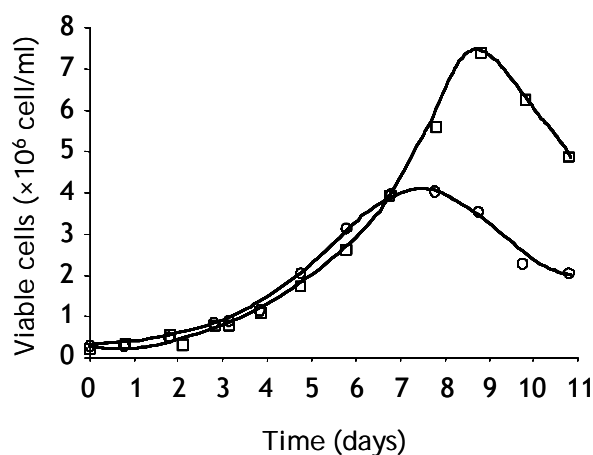


Figure 2.1. Growth curves of human 293 cells obtained in two different culture media: (o) CD 293 and ( $\square$ ) EX-Cell.

### 3.2. Effect of media on AdV productivity

Since EX-Cell medium allowed the higher maximum cell density, the effect upon AdV productivity at high CCI was evaluated and compared with CD 293 medium. As shown in Figure 2.2, when a CCI of  $1 \times 10^6$  cell/ml was used,

both culture media were similar in their ability to “support” AdV production. The specific productivity in human 293 cells using CD 293 medium was not significantly affected when a CCI of  $2 \times 10^6$  cell/ml was used, while a 10 fold decrease was observed when EX-Cell medium was used. For a CCI of  $3 \times 10^6$  cell/ml no effective AdV production was observed for EX-Cell, and for CD 293 a 1 log decrease (as compared with CCI of  $1 \times 10^6$  cell/ml) was observed, confirming the CD 293 medium as a good choice for AdV production at high cell densities (Ferreira et al., 2005b).

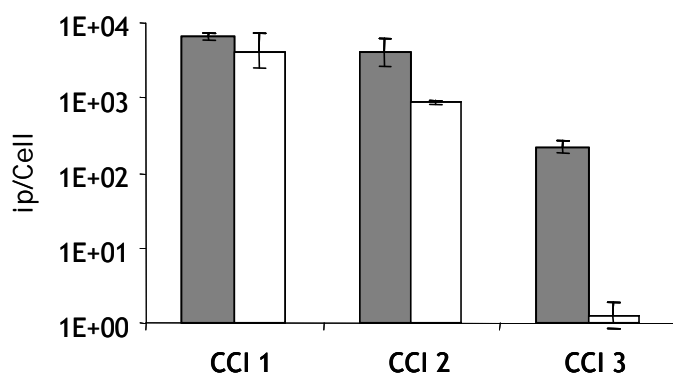


Figure 2.2. Cell specific productivity in CD 293 (■) and EX-Cell (□) at CCIs of 1, 2 and  $3 \times 10^6$  cell/ml. Error bars represent the propagated error considering one SD of quadruplicate sample assay for two samples.

The decrease in cell specific productivity obtained at a CCI of  $3 \times 10^6$  cell/ml in CD 293 and EX-Cell media might be due to the lack of an essential nutrient or growth factors or to the byproducts accumulation. As stated in Chapter III, Part 1, glutamine, threonine and glucose are limiting nutrients in CD 293 medium at CCI of  $3 \times 10^6$  cell/ml, although no improvement on AdV productivity was achieved after the addition of these nutrients at the time of infection; on the other hand, ammonia was at an inhibitory concentration for infection at this CCI. In this present study, following ammonia production for both media, it was observed that at  $3 \times 10^6$  cell/ml



ammonia concentration was 1.8 times higher in EX-Cell medium than in CD 293 (Figure 2.3), supporting that ammonia is one of the main contributor for the “cell density effect”.

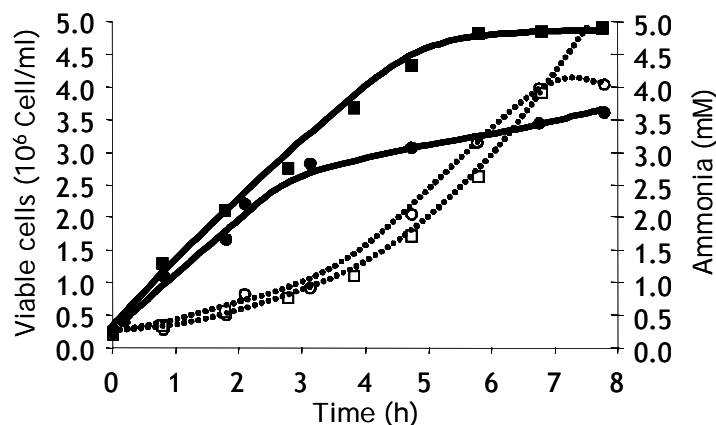


Figure 2.3. Ammonia production (full lines) during cell growth (broken lines) in CD 293 (●, ○) and in EX-Cell (■, □).

### 3.3. Effect of osmolality increase on cell growth and AdV productivity

The effect of increasing osmolality on human 293 cell growth was evaluated by increasing it to 330, 380, 430 and 480 mOsm from original values of 230 mOsm at the inoculation time and when the cells reached a concentration of  $1 \times 10^6$  cell/ml (concentration where the cell specific productivity is maximal). As shown in Table 2.1, decreases on specific growth rate and maximum viable cell concentration were observed when the osmolality was increased at the inoculation time, leading to growth inhibition at the maximum osmolality studied (480 mOsm), whereas osmolality increases at  $1 \times 10^6$  cell/ml had a lesser effect upon both specific growth rate and maximum viable cell concentration.

Table 1.1. Specific growth rates ( $\mu$ ) and maximum viable cell concentration ( $X_{\max}$ ) obtained at different osmolalities.

	$\mu$ ( $h^{-1}$ )	$X_{\max}$ ( $\times 10^6$ cell/ml)
Control	$0.019 \pm 0.001$	$3.81 \pm 0.19$
At inoculation time		
330i	$0.018 \pm 0.001$	$2.81 \pm 0.14$
380i	$0.013 \pm 0.001$	$2.18 \pm 0.11$
430i	$0.009 \pm 0.001$	$0.94 \pm 0.05$
480i	-	-
At $1 \times 10^6$ cell/ml		
330m	$0.015 \pm 0.001$	$3.08 \pm 0.15$
380m	$0.011 \pm 0.001$	$3.20 \pm 0.16$
430m	$0.009 \pm 0.001$	$2.23 \pm 0.11$
480m	$0.004 \pm 0.001$	$1.61 \pm 0.08$

i- osmolality increased at inoculation time;

m- osmolality increased when the cells reached  $1 \times 10^6$  cell/ml.

The effect of increasing osmolality on AdV productivity at a cell concentration of  $1 \times 10^6$  cell/ml was evaluated by increasing the osmolality at the inoculation time or at the infection time. The infection of the cells with an increased initial osmolality of 430 mOsm and 480 mOsm was not evaluated because the cells did not reach the concentration of  $1 \times 10^6$  cell/ml. As shown in Figure 2.4, osmolality values up to 430 mOsm did not affect AdV productivity but an increase in osmolality for 480 mOsm at infection time caused a reduction on cell specific productivity of two orders of magnitude.

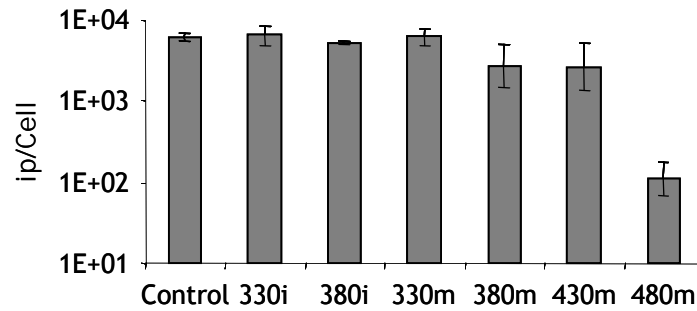


Figure 2.4. Cell specific productivity obtained at infection at a CCI of  $1 \times 10^6$  cell/ml, after increases in osmolality. i: at inoculation time; m: when the cells reached  $1 \times 10^6$  cell/ml. Error bars represent the propagated error considering one SD of quadruplicate sample assay for two samples.

From the data obtained in this work it can be concluded that CD 293 medium is the better medium for AdV production in human 293 cells at high CCI when compared with EX-Cell medium. Possibly the main reason for such a result is the higher ammonia production registered in EX-Cell medium. It can also be concluded that increases in osmolality at the time of infection do not have any negative effect in terms of cell growth and AdV productivity as long as osmolality after infection is kept below 430 mOsm. Thus, fed-batch supplementation during the growth and production phases does not have any effect in terms of osmolality, since such values are difficult to achieve. Moreover, contrary to the increase in PTP1C protein production observed by Nadeau et al. (1996) on these cells with increased osmolality obtained by NaCl addition, in this work the cell specific productivity was not enhanced. Furthermore, the addition of NaCl may affect cell behaviour, since the imbalance in osmotic pressure increase the uptake of  $\text{Na}^+$  and, consequently, alter cell metabolism. However, also in this case, no effect on AdV productivities was registered.

#### 4. ACKNOWLEDGEMENT

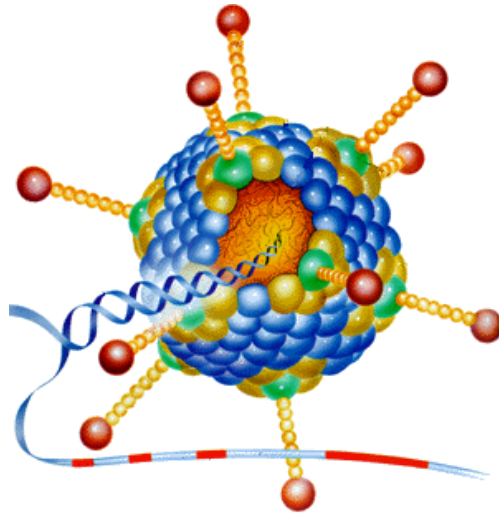
The authors are grateful to Dr Tom Barret (Institute for Animal Health - Pirbright, UK) for providing the recombinant adenovirus. The authors acknowledge the financial support received from the European Commission (Project ICF599A4PR01) and from Fundação para a Ciência e Tecnologia - Portugal (Project POCTI/BIO/46515/2002 and student grant SFRH/BD/10614/2002).

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## CHAPTER III

### Medium Limitations





## PART 1

Effect of refeed strategies and non-ammoniagenic medium on adenovirus production at high cell densities

Ferreira T.B., Ferreira A.L., Carrondo M.J.T., Alves P.M. (2005) J. Biotechnol., 119:272-280.

## Abstract

Recombinant adenoviruses became one of the vectors of choice for delivery and expression of foreign proteins for gene therapy and vaccination purposes. Nevertheless, the production of adenovirus is currently limited by the so-called “cell density effect”, i.e., a drop in cell specific productivity concomitant with increased cell concentration at infection (CCI).

This work describes the characterisation and optimisation of the infection process in order to improve recombinant adenovirus type 5 yields at high cell densities. For that purpose, 293 cells adapted to suspension were grown in 2 L bioreactors and infected at different cell concentrations, using different refeed strategies, while evaluating cell metabolism. The consumption of amino acids is enhanced during infection, although no amino acid limitation was detected for cells infected at concentrations in the range of  $2 \times 10^6$  cell/ml, for which the highest volumetric productivity was obtained in batch mode. Conversely, infecting at cell concentrations in the range of  $3 \times 10^6$  cell/ml led to complete depletion of glucose, glutamine and threonine before the optimal harvesting time, a significant decrease in volumetric productivity being observed; the effect of amino acids and glucose addition at infection time on cell specific and volumetric productivity of adenovirus was assessed, no improvement on adenovirus production being achieved. The effect of ammonia, present in high concentrations at  $3 \times 10^6$  cell/ml, was evaluated and seem to be detrimental; an 1.8 fold increase on adenovirus volumetric productivity was obtained for infections performed at  $3 \times 10^6$  cell/ml when non-ammoniagenic medium was used.

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## 1. INTRODUCTION

Delivery of genes to somatic tissue for the prevention and treatment of a wide range of genetic and acquired diseases is a promising route for therapy. The added genes can provide a missing function, modulate the immune response or initiate cell suicide in the presence of certain drugs. Since recombinant adenoviruses (AdV) are highly efficient at gene transfer for a broad spectrum of cell types and species they became one of the vectors of choice for gene delivery and expression of foreign proteins in gene therapy and vaccination (Rea et al., 1999; Mountain 2000). Consequently, the market requirements for AdV are increasing, driving the search for new methodologies for large scale production of concentrated vectors with warranted purity and efficacy.

Different approaches for the improvement of production and purification processes of these viral vectors have been reported (Huyghe et al., 1995; Nadeau et al., 1996; Iyer et al., 1999; Blanche et al., 2000). Factors such as the construction of the vector itself, the host cell line and the culture medium can affect AdV production rates (Eloit and Adam 1995; Iyer et al., 1999; Nadeau and Kamen 2003). Effects of pH, pCO<sub>2</sub> and temperature on AdV production have also been reported (Xie et al., 2002; Jardon and Garnier 2003). Currently, AdV production at high cell specific productivity is limited to low cell densities, in the range of  $1 \times 10^6$  cell/ml (Kamen and Henry 2004). This "cell density effect" has been considered by several authors as a consequence of nutrient limitations or accumulation of byproducts. The understanding of these limiting factors will contribute to design a robust and cheap process for AdV production at high cell densities, by adding the essential nutrients or using a new medium formulation that allows the infection at higher cell densities maintaining the specific cell productivity. Although metabolic flux analysis has been used to characterize the effect upon cell metabolism of various environmental conditions the nature of the factors limiting AdV productivities at high cell

densities remains unknown (Nadeau et al., 2000a; Nadeau et al., 2000b; Nadeau et al., 2002).

Different strategies for improvement have been proposed: Garnier et al. (1994) demonstrated that medium replacement at 0 hours post infection (hpi) and the addition of glucose at 24 hpi together with periodical pH adjustments, allowed a sustained maximum cell specific productivity at  $1.6 \times 10^6$  cell/ml whereas, Nadeau et al. (1996) further improved this strategy by also adding essential amino acids at 24 hpi thereby stabilising AdV volumetric productivity at cell densities just above  $2 \times 10^6$  cell/ml. At production scale, medium exchange will increase the cost of the final product, which is even more critical when the final product is to be used in the veterinary field. Perfusion mode operation has been attempted as a means to control the culture environment and remove toxic byproducts (Henry et al., 2004; Kamen and Henry 2004); nevertheless, the cell specific productivity could only be maintained by infecting cells at densities up to  $3 \times 10^6$  cell/ml using high perfusions rates of 2 reactor volumes per day, at 2 days post infection, a very costly proposition.

The accumulation of ammonia and lactate, major products from glutamine and glucose metabolism, has been demonstrated to diminish mammalian cell growth and influence cell metabolism and protein productivity (Cruz et al., 2000). Attempts have been made to control cell metabolism and reduce the production of these metabolites, such as: i) maintaining low concentrations of glucose and glutamine to shift cell metabolism towards more efficient states (Cruz et al., 1999), or ii) adapting the cells to grow in media with other carbon and nitrogen sources like galactose and glutamate (Altamirano et al., 2000). Specifically, for 293 cells often utilised for AdV production, lactate at 20 mM reduced cell viability to 50% with a much reduced effect on product, whereas ammonia at 1 mM already inhibited cell growth; under these conditions the effects upon AdV production were not identified (Nadeau et al., 1996).

In this work, factors limiting the AdV production at high cell densities were

investigated. Refeed strategies and the adaptation to non-ammoniagenic media were evaluated in order to understand the metabolic requirements when infection is done at high cell densities and to overcome the so called "cell density effect", thus permitting the achievement of higher volumetric productivities for AdV. A first generation AdV vector was employed.

## 2. MATERIALS AND METHODS

### 2.1. Cell line and medium

293 cells, purchased from ATCC (ATCC-CRL-1573), were adapted to suspension and grown in commercially available serum and protein free medium, CD 293, supplemented with 4 mM of glutamine (all from Invitrogen, Glasgow, UK) at a humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C in shake flasks (corning, NY). Cells were routinely propagated twice a week with an inoculum of  $0.5 \times 10^6$  cell/ml.

### 2.2. Infection in shake flasks

Infection was performed in 125 ml shake flasks with final volumes of 40 ml in a humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C. All the infections were done using a multiplicity of infection (MOI) of 10 and AdV harvested at 48 hpi (see Annex A).

### 2.3. Stirred tank studies

Bioreaction studies were performed in a 2 L bioreactor (Braun, Melsungen, Germany). The agitation rate was maintained at 110 rpm ( $N_{Re} = 6800$ ); pH was controlled at 7.2 by aeration with a CO<sub>2</sub> gas-mixture and NaOH 0.2 M; the dissolved oxygen was controlled at 80% air saturation. All the infections were done using a MOI of 10 and AdV harvested at 48 hpi (see Annex A). For

the fed-batch operation mode, threonine, glutamate, amino acids cocktail (RPMI 1640 50×) (all from Sigma-Aldrich, St. Louis, MO), glutamine and glucose (Merck, Darmstadt, Germany) solutions were used.

#### 2.4. Virus culture samples preparation

A replication-defective AdV derived from type 5 AdV was kindly provided by Dr. Tom Barret (IAH-Pirbright, UK). Infected 293 cells were harvested at 48 hpi and centrifuged at 1000g for 10 min. at 4°C. The resulting cell supernatant was distributed into small aliquots and stored at -85°C for the evaluation of extracellular virus. The resulting cell pellet was resuspended in a known volume of Tris buffer 10 mM, pH 8.0 supplemented with 2 mM MgCl<sub>2</sub> and 0.1% Triton. Cells were disrupted by vortexing for 1 minute and cell debris removed by centrifugation at 3000g for 10 min. at 4°C. The resulting cell lysate supernatant was dispensed into small aliquots and stored at -85°C for quantification of intracellular viral content.

#### 2.5. Analytical Methods

Cell concentration and viability were determined by counting cells on a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany) using the trypan blue (Invitrogen) dye exclusion method.

AV titration was performed by the end-point dilution method (TCID<sub>50</sub>) using 96 well plates and 293 cells. The titer was determined according to the method of Spearman and Kraber, as described elsewhere (Darling et al., 1998).

Glucose and lactate were analyzed using a YSI Multiparameter Bioanalytical System Model 7100 MBS (Yellow Springs, USA).

Ammonia was quantified enzymatically using a UV test number 1112732035 (Boehringer Mannheim, R-Biopharm AG, Germany).

Complete analysis of amino acid composition was done by HPLC using a Pico-Tag (Waters) system. Amino acids were derivatized with the phenyl-isothiocyanate method (Matsudaira 1990). The Pico-Tag system consisted of a Nova-Pak C18 column (WAT 011695), a Waters 510 pump, and automatic injector Waters 717 and a tunable Absorbance Detector 486. Data acquisition was performed on a PC using software from Millenium.

### 3. RESULTS AND DISCUSSION

#### 3.1. Effect of CCI on AdV production

The effect of CCI on the final AdV production yields was evaluated in 2 L bioreactor. Infection at CCI  $1 \times 10^6$  cell/ml (control),  $2 \times 10^6$  cell/ml and  $3 \times 10^6$  cell/ml with or without complete media exchange at the time of infection was compared (Fig. 3.1.1). As can be observed, cell specific productivity is not significantly affected at CCI  $2 \times 10^6$  cell/ml, when compared with the control, leading to a 1.3 fold increase on AdV volumetric productivity. However, one log decrease on cell specific productivity was observed at CCI  $3 \times 10^6$  cell/ml.

Medium exchange at the time of infection and the addition of glucose and essential amino acids at 24 hpi has been reported to allow a sustained maximum specific productivity just above  $2 \times 10^6$  cell/ml (Nadeau et al., 1996). Figure 3.1.1 shows that a single medium exchange at the time of infection is enough to maintain the cell specific productivity at the maximum CCI of  $3 \times 10^6$  cell/ml, for which the highest volumetric productivity was obtained. The results obtained are improvements on those reported in the literature, suggesting the serum and protein free medium used in this study (CD 293) to be a good choice for AdV production at high cell densities. Nevertheless, at production scale, a medium exchange could make the final product cost prohibitive. To determine how these results



are related to the metabolism of the cells, the kinetics of nutrient consumption and metabolite production were evaluated.

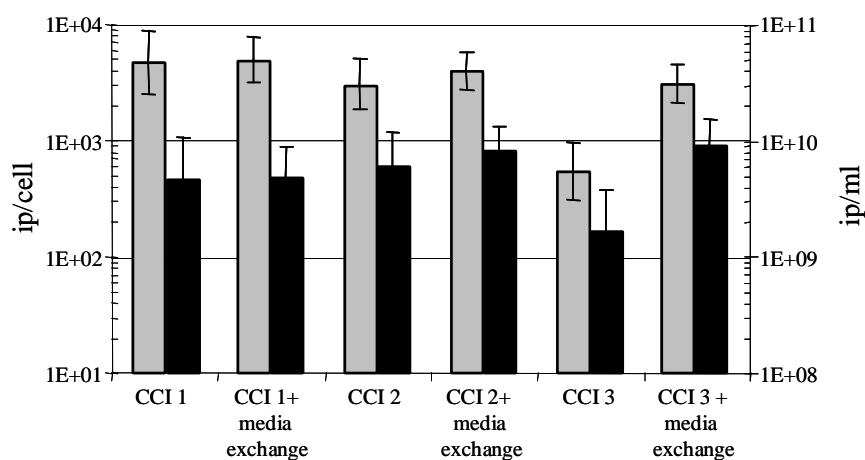


Figure 3.1.1. Effect of media exchange and CCI in cell specific (■) and AdV volumetric (■) productivities. CCI 1, CCI 2 and CCI 3 correspond to 1, 2 and  $3 \times 10^6$  cell/ml at time of infection, respectively.

### 3.2. Cell metabolism evaluation at different CCIs

Cell metabolism was assessed before and after infection at CCI  $1 \times 10^6$  cell/ml and  $3 \times 10^6$  cell/ml. After infection at CCI  $1 \times 10^6$  cell/ml, increases of 1.3 fold in glutamine and 1.8 fold in glucose specific consumption rates were observed, concomitant with increases of 1.2 fold in ammonia and 3.9 fold in lactate specific production rates (data not shown). No significant alteration in the molar ratio of ammonia produced/glutamine consumed was observed, whereas the molar ratio of lactate produced/glucose consumed increased 2.3 times (Table 3.1.1). Elias et al. (2003) observed that for 293 cells, the flux of glucose into the TCA cycle and its subsequent utilization is limited as a result of the lack of certain key enzymes in this pathway. This may be the explanation for the significant increase in the molar ratio of lactate produced/glucose consumed observed in our

experiments, since after infection the glucose uptake increases as a result of energy requirements; however, the flux of glucose into the TCA cycle seems limited as the fermentation takes place, leading to lactate accumulation.

Table 3.1.1. Molar ratios of ammonia produced/glutamine consumed and lactate produced/glucose consumed in non infected cells, cells infected at CCI  $1 \times 10^6$  cell/ml and CCI  $3 \times 10^6$  cells/ml.

	Non infected	CCI 1	CCI 3
$Y_{\text{ammonia/glutamine}}$	0.67	0.59	0.30
$Y_{\text{lactate/glucose}}$	0.78	1.83	0.50

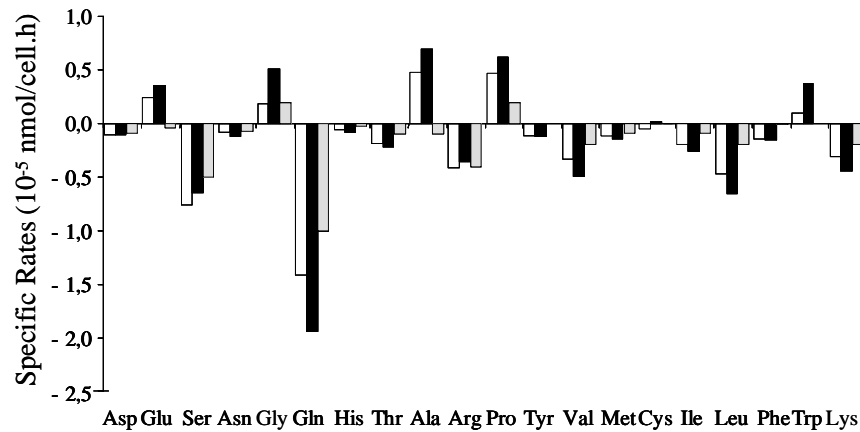


Figure 3.1.2. Effect of infection and CCI in amino acid metabolism. The bar correspond to specific consumption and production rates of amino acids in non infected cells (□), cells infected at CCI 1 (■) and  $3 \times 10^6$  cell/ml (▒).

For almost all amino acids, increases in specific consumption and production rates were observed after infection at CCI  $1 \times 10^6$  cell/ml (Fig. 3.1.2). However, when the infection was performed at CCI  $3 \times 10^6$  cell/ml, a decrease in amino acids specific consumption rates was observed while

some of the usually produced amino acids started to be consumed. Moreover, significant decreases in glucose specific consumption rate, as well as in ammonia and lactate specific production rates, were observed for the infection at this CCI (data not shown). Following the decay in cell viability after infection (Fig. 3.1.3) a significant decrease was observed at 24 hpi for CCI  $3 \times 10^6$  cell/ml, while at CCI  $1 \times 10^6$  cell/ml only after 72 hpi the cell decay becomes apparent, this being the time, according to the literature, corresponding to an infection cycle (Nadeau and Kamen 2003).

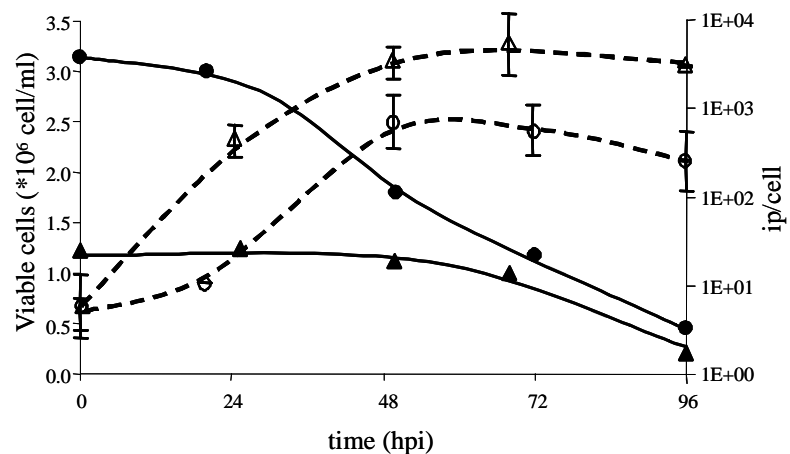


Figure 3.1.3. Effect of CCI in cell viability (full symbols) and in AdV productivity (empty symbols). CCI  $1 \times 10^6$  cell/ml are represented as triangles and CCI  $3 \times 10^6$  cell/ml as circles.

Figure 3.1.3 confirms cell specific production rate to be lower at CCI  $3 \times 10^6$  cell/ml, as expected. These results support the claim that the “cell density effect” is a consequence of nutrient limitations or accumulation of byproducts. Moreover the consumption of the amino acids that are usually produced is a means for the cells to balance the lack of nutrients. From the analysed nutrients, it was observed that glutamine, threonine and glucose are totally consumed before the optimal harvesting time that corresponds to 48 hpi (Fig. 3.1.4); however, the decrease in the metabolic activity

started at the time of infection and so did the AdV productivity. It can be further observed that lactate and ammonia concentrations were high at the time of infection: 17.4 mM and 1.5 mM, increasing to 22 mM and 1.8 mM, respectively, at 48 hpi. Thus, limitation of nutrients together with a toxic effect promoted by the accumulation of byproducts seems responsible for the significant decrease on AdV production yields observed at the highest CCI.

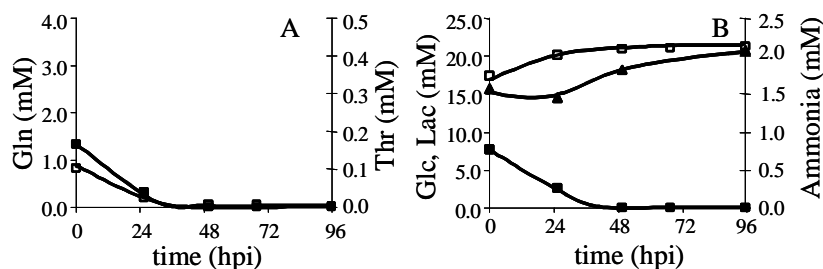


Figure 3.1.4. (A) Glutamine (Gln) (■) and threonine (Thr) (□) consumption and (B) glucose (Glc) (■) consumption and lactate (Lac) (□) and ammonia (▲) production after infection at CCI  $3 \times 10^6$  cell/ml. 4.0, 0.5 and 25.0 mM (maximum value of y axis scale) are the initial medium concentration for glutamine, threonine and glucose respectively.

### 3.3. AdV production with a refeed strategy

Since glutamine, threonine and glucose were found to be limiting nutrients at CCI  $3 \times 10^6$  cell/ml, two different fed-batch operation modes were tested with the addition of either (1) threonine, glutamine and glucose or (2) an amino acid cocktail and glucose at the time of infection; both were compared against a batch control mode (Fig. 3.1.5). Surprisingly, no improvement on cell specific and AdV volumetric productivities was obtained for either strategy. A significant increase on ammonia accumulation for both strategies was observed, when compared with the

batch control, associated with the glutamine addition (data not shown). These results confirm that glutamine, threonine and glucose limitation are not the only limiting factors on AdV production, since the refeeding of these nutrients was not enough to improve AdV production yields at CCI  $3 \times 10^6$  cell/ml.

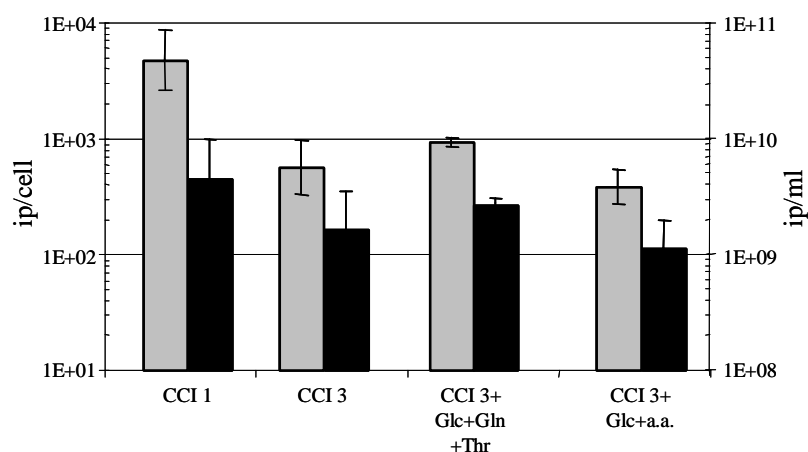


Figure 3.1.5. Effect of refeed strategies in cell specific (■) and AdV volumetric (■) productivities. CCI 1 and CCI 3 correspond to 1 and  $3 \times 10^6$  cell/ml at time of infection, respectively. Fed-batch operations modes were performed with glucose (Glc), glutamine (gln) and threonine (thr) or Glc and amino acids cocktail (a.a.) refeed at the time of infection.

### 3.4. AdV production in non-ammoniagenic media

Table 3.1.2. Specific growth rates ( $\mu$ ) and maximum total cell number ( $X_{max}$ ) obtained for cells growing in glutamine supplemented medium and in non ammoniagenic medium.

	Glutamine	Non-ammoniagenic
$\mu$ ( $h^{-1}$ )	0.018	0.014
$X_{max}$ (cell/ml)	$5.5 \times 10^6$	$4.5 \times 10^6$

An important evidence from this study was the high ammonia concentration observed at CCI  $3 \times 10^6$  cell/ml (1.5 mM) (Fig. 3.1.4),

considered toxic for cell growth (Nadeau et al., 1996); however the toxic effect of this byproduct on AdV production is not known.



Figure 3.1.6. Cell specific consumption and production rates of amino acids in cells growing in glutamine supplemented medium (□) and non-ammoniagenic medium (■).

Ammonia was shown to induce cytoplasm acidification which can affect AdV stability (McQueen and Bailey 1991). Thus, in order to understand the effect of this byproduct on AdV production yields, 293 cells were adapted to grow in CD 293 medium in which glutamine was replaced by glutamate (from now on referred as non-ammoniagenic medium). As for other cell lines (Christie and Butler 1999), a prolonged lag phase, reduced growth rates and lower maximum cell densities were observed (Table 3.1.2). As expected, ammonia specific production rate was significantly reduced, from  $4 \times 10^{-9}$  mmol/cell.h for cells grown in glutamine supplemented medium to  $0.09 \times 10^{-9}$  mmol/cell.h for cells growing in non-ammoniagenic medium; an increase in all amino acids specific consumption rate was also observed, particularly glutamate, which is produced in cells growing in glutamine supplemented media, and serine (Fig. 3.1.6). Furthermore, it was observed that the increase in threonine specific consumption rate led

to the depletion of this amino acid and a refeed had to be done at  $2 \times 10^6$  cell/ml. Comparing with glutamine supplemented medium, no significant variations were observed in glucose specific consumption and lactate production rates for non infected cells.

Table 3.1.3. Molar ratios of lactate produced/glucose consumed in non infected cells, cells infected at CCI  $1 \times 10^6$  cell/ml and CCI  $3 \times 10^6$  cells/ml for 293 cells adapted to non-ammoniagenic medium.

	Non infected	CCI 1	CCI 3
$Y_{\text{lactate/glucose}}$	0.73	1.42	0.55

293 cells adapted to non-ammoniagenic medium were infected at CCI  $1 \times 10^6$  cell/ml and CCI  $3 \times 10^6$  cell/ml. After infection at CCI  $1 \times 10^6$  cell/ml, while glucose specific consumption rate increased 1.3 times, an increase of 2 fold in lactate specific production rate was observed (data not shown). It seems that glucose was used in a more energetic way, since the molar ratio of lactate produced/glucose consumed increased 1.9 times (Table 3.1.3), less than the observed value when glutamine containing medium was used, and less lactate was accumulated. Comparing with infection of cells growing in glutamine supplemented medium, 1.8 times higher AdV volumetric productivities were observed at CCI  $3 \times 10^6$  cell/ml than at CCI  $1 \times 10^6$  cell/ml (Fig. 3.1.7); although there was a drop of the cell specific productivity, this was not so significant as the corresponding drop for glutamine containing medium, being 39% and 88% respectively. The final ammonia concentration was approximately 10 times lower at the highest CCI for the non-ammoniagenic medium than that observed for the glutamine supplemented medium, at 0.2 mM and 2 mM respectively; also the final lactate concentration was 1.8 times lower (12 mM) when the infection was performed in the non-ammoniagenic medium at the highest CCI, below to the reported toxic concentrations (data not shown).

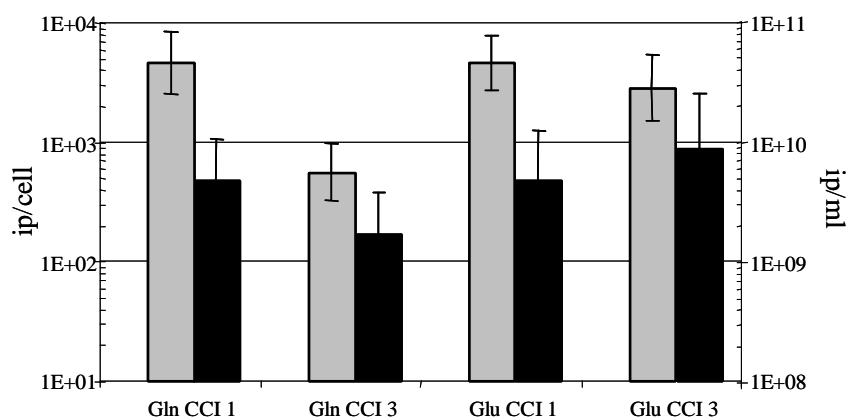


Figure 3.1.7. Effect of medium supplement in cell specific (■) and AdV volumetric (■) productivities. Gln and Glu correspond to glutamine and glutamate supplemented medium and CCI 1 and CCI 3 correspond to 1 and  $3 \times 10^6$  cell/ml at time of infection, respectively.

These results suggest an inhibitory effect on AdV production by ammonia accumulation and confirmed that the use of non-ammoniagenic medium improved AdV volumetric productivity. However, the ammonia accumulation seems not to be the only inhibitory factor on AdV production, since in optimal condition, a 3 times increase on AdV volumetric productivity was to be expected at CCI  $3 \times 10^6$  cell/ml, when only an 1.8 fold increase was obtained, corresponding to the highest decrease in cell specific productivity indicated above.

#### 4. CONCLUSIONS

The present study shows that by using CD 293 medium the AdV production reaches the highest volumetric productivity reported for an infection performed at CCI of  $2 \times 10^6$  cell/ml in batch mode. Moreover, AdV productivity can be improved even at a CCI of  $3 \times 10^6$  cell/ml with a single



CD 293 medium exchange at the time of infection. Nevertheless, for scaled up processes, a medium exchange step is not cost effective.

Glutamine, threonine and glucose were found to be limiting nutrients at CCI of  $3 \times 10^6$  cell/ml, although no improvement on AdV productivity could be achieved after the addition of these nutrients at the time of infection. On the other hand, ammonia was at an inhibitory concentration for infection at this CCI. By adapting the cells to non-ammoniagenic medium, an 1.8 fold increase in AdV volumetric productivity at CCI  $3 \times 10^6$  cell/ml was obtained, although the "ideal" situation would have been an increase of 3 times at this CCI; thus, ammonia is an important parameter to be considered for infection at high cell densities, but not the only.

Future improvements to the culture process may come from analysis of other nutrients than the usual glucose, lactate, ammonia and amino acids. A cocktail of different nutrients are present in the culture media that may have an important impact on AdV production as vitamins, lipids, hormones and growth factors (see Annex B). Combination of these measurements may lead to the design of a new strategy permitting a higher cell specific and, consequently, AdV volumetric productivity.

## 5. ACKNOWLEDGEMENT

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## PART 2

Effect of ammonia production on intracellular pH: consequent effect on adenovirus vector production

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## Abstract

Recombinant adenoviral vectors (AdV) have proven to be highly efficient for the delivery and expression of foreign genes in a broad spectrum of cell types and species both for vaccination and gene therapy in a number of specific applications.

In this study, the effect of ammonia production on intracellular pH ( $pH_i$ ) and consequently inhibition of AdV production at high cell densities is assessed. Different specific ammonia production rates were obtained for 293 cells adapted to grow in glutamate supplemented medium (non-ammoniagenic medium) as compared with 293 cells growing in glutamine supplemented medium (ammoniagenic medium);  $pH_i$  was observed to be lower during cell growth and AdV production at both high and low CCI in the ammoniagenic medium, where the specific ammonia production rate is higher. In addition, after infection at CCI of  $3 \times 10^6$  cell/ml, the cell viability decreased significantly in the ammoniagenic medium, attributed to the activation of an acidic pathway of apoptosis. Furthermore, AdV DNA was observed to be degraded at the observed  $pH_i$  in the ammoniagenic medium, decreasing significantly the amount of AdV DNA available for encapsidation. To elucidate the  $pH_i$  effect upon AdV production, 293 cells were infected at a CCI of  $1 \times 10^6$  cell/ml in the non-ammoniagenic medium with a manipulated  $pH_i$  as observed at the time of infection at CCI of  $3 \times 10^6$  cell/ml in the ammoniagenic ( $pH_i$  7.0) and non-ammoniagenic ( $pH_i$  7.3) media; AdV volumetric productivities were observed to be lower when the cells were exposed to the lower  $pH_i$ . Thus, the importance of controlling all the factors contributing to  $pH_i$  on AdV production, such as ammonia production, has been established.

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## 1. INTRODUCTION

Recombinant adenoviral vectors (AdV) have proven to be highly efficient for the delivery and expression of foreign genes in a broad spectrum of cell types and species, in a number of specific applications (Volpers and Kochanek, 2004; Ferreira et al., 2005a). Thus, as the market needs for AdV are increasing, methodologies for production of concentrated AdV are needed. Although 293 cells can be grown up to  $8 \times 10^6$  cell/ml in batch systems (Ferreira et al., 2005c), AdV production at high cell densities is limited by the so-called “cell density effect”, i.e., a drop in cell specific productivity concomitant with increased cell concentration at infection (CCI) for values above  $1 \times 10^6$  cells/ml (Nadeau and Kamen, 2003; Kamen and Henry, 2004; Ferreira et al., 2005b). Although this effect has been attributed mainly to nutrient limitations and/or byproducts accumulation, the exact nature of the factors limiting AdV productivities at high CCIs remains unknown. Previous studies have indicated that medium replacement at the time of infection and the addition of glucose and amino acids at 24 h post infection (hpi) together with periodic pH adjustments, allows for high cell specific productivity at cell densities in the range  $2 \times 10^6$  to  $3 \times 10^6$  cells/ml (Garnier et al., 1994; Nadeau et al., 1996). Our group has shown that ammonia is an important parameter for infection at high cell densities, since, by adapting 293 cells to non-ammoniagenic medium, a 5 fold increase on cell specific productivity could be obtained at a CCI of  $3 \times 10^6$  cell/ml (Ferreira et al., 2005b). However, the mechanisms by which ammonia inhibits AdV production remain unknown.

Ammonia derives mainly from deamination of glutamine, whose concentration in the culture medium is usually 5 to 20 fold higher than that of other amino acids (Eagle, 1955). Complete catabolism of glutamine produces 2 mol of ammonia per mol of glutamine. Other minor sources of ammonia include the spontaneous degradation of glutamine (Ozturk and Palsson, 1990; Arai et al., 1999) and catabolism of other amino acids.



Several mechanisms of ammonia toxicity have been described in animal cells, including: disturbance of electrochemical gradients (Mirabet et al., 1997); inhibition of enzyme reactions; changes in intracellular pH ( $\text{pH}_i$ ) (McQueen and Bailey, 1990); increased demand for energy maintenance (Martinelle and Haggstrom, 1993); inhibition of cell growth; and perturbed processing and secretion of proteins and apoptosis induction (Cruz et al., 2000).

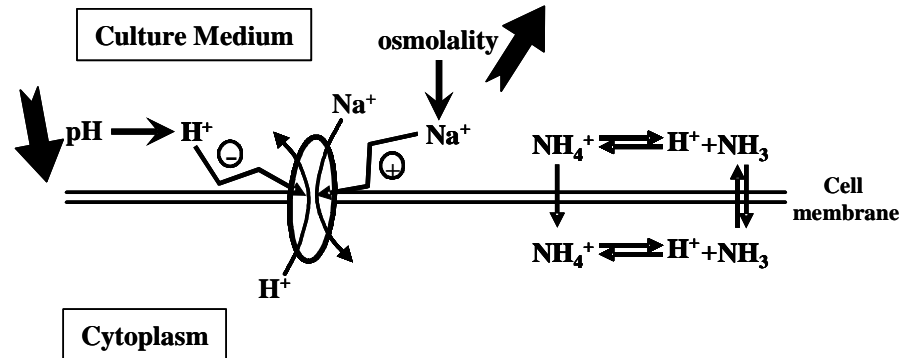


Figure 3.2.1 Mechanisms influencing  $\text{pH}_i$  at decreased medium pH, increased ammonium ion concentration, and increased medium osmolality (adapted from Cherlet and Marc (1998)).

In the case of  $\text{pH}_i$ , ammonia can disturb the optimal cytoplasmatic pH by forming a "proton shuttle" (represented schematically in Figure 3.2.1): briefly, the intracellularly originated ammonium ions ( $\text{NH}_4^+$ ) are rapidly excreted by passive diffusion, under the form of ammonia ions ( $\text{NH}_3$ ), to the extracellular milieu with concomitant accumulation inside the cell of one proton ( $\text{H}^+$ ) per  $\text{NH}_3$  molecule released. This excretion is facilitated by the negative charge of the membrane potential and the gradient of ammonia ions across the cell membrane. Once outside the cell,  $\text{NH}_4^+$  can enter the cell again propelled by the large electrical gradient due to the highly charged membrane and leave the cell again under the  $\text{NH}_3$  form; this cycle can be started over and over again (McQueen and Bailey, 1990; Wu et

al., 1993). If this proton load is higher than the overall cell capacity to excrete it, the  $\text{pH}_i$  decreases. If ammonium ions ( $\text{NH}_4^+$ ) are added to the culture medium,  $\text{NH}_3$  diffusion into cells predominates, leading to cell alkalinization. In addition to ammonia,  $\text{pH}_i$  can also be affected by extracellular pH ( $\text{pH}_e$ ) and osmolality (Wu et al., 1993) (Figure 3.2.1). However, these are not so critical for AdV production because  $\text{pH}_e$  can be controlled in the bioreactors and high osmolality leads to cytoplasmic alkalinization (Frelin et al., 1988).

It is known that AdVs have a low stability at acidic pH (Evans et al., 2004). Also, several studies describe the effect of pH on the stability of mammalian DNA namely on the correlation between  $\text{pH}_i$  and the activity of the nuclear enzymes DNase I and DNase II (Barry and Eastman, 1993; Gottlieb et al., 1996; Matsuyama et al., 2000; Lagadic-Gossmann et al., 2004). However, the mechanisms describing how pH affects AdV DNA are still unknown.

In this work, we report on the effect of ammonia accumulation on  $\text{pH}_i$  and its consequent effect on AdV production. For that purpose 293 cells adapted to grow in suspension in glutamine supplemented medium (ammoniagenic medium) and in glutamate supplemented medium (non-ammoniagenic medium) were compared in terms of intracellular pH and AdV production.

## 2. MATERIALS AND METHODS

### 2.1. Cell line and medium

Anchorage-dependent 293 cells, purchased from ATCC (ATCC-CRL-1573), were cultured in Minimum Essential Medium (MEM) supplemented with 5% heat-inactivated (56 °C, 30 min.) Foetal Bovine Serum (FBS) and 2 mM of glutamine (all from Invitrogen, Glasgow, UK) using a humidified atmosphere of 5%  $\text{CO}_2$  in air at 37°C. The anchorage-dependent 293 cells

were further adapted to grow in suspension in commercially available serum and protein free medium, CD 293, supplemented with 4 mM of glutamine (ammoniagenic medium) (all from Invitrogen, Glasgow, UK) or glutamate (non-ammoniagenic medium) (Sigma-Aldrich, St. Louis, MO) in an humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C in shake flasks (Corning, NY). Cells were routinely propagated, twice a week, with an inoculum of 0.5×10<sup>6</sup> cell/ml.

## 2.2. Infection in shake flasks

A replication-defective AdV derived from human Ad5 was kindly provided by Dr. Tom Barret (Institute for Animal Health-Pirbright, UK). Infection was made in CD 293 medium when the cells reached a concentration (CCI) of 1×10<sup>6</sup> cell/ml or 3×10<sup>6</sup> cell/ml using a multiplicity of infection (MOI) of 10 (see Annex A) in 125 ml shake flasks with a working volume of 40 ml in a humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C. Infected 293 cells were harvested at 48 h post infection (hpi) (see Annex A), earlier determined as the optimal harvesting time (Ferreira et al., 2005b). In the studies where the pH<sub>i</sub> was changed, 293 cells were grown with non-ammoniagenic medium supplemented with 30 mM KCl and 20 µl of 1 mM of the strong carboxylic ionophore nigericin (Pressman, 1976) (Invitrogen, Glasgow, UK) in absolute ethanol or 30 mM KCl and 20 µl of absolute ethanol (control).

## 2.3. Virus culture samples preparation

Infected 293 cells were harvested at 48 hpi and centrifuged at 1000g for 10 min. at 4°C. The resulting cell supernatant was distributed into small aliquots and stored at -85°C for extracellular virus titration. The resulting cell pellet was resuspended in a known volume of Tris buffer 10 mM, pH 8.0 supplemented with 2 mM MgCl<sub>2</sub> (Merck, Darmstadt, Germany) and 0.1% (v/v) Triton (Sigma-Aldrich, St. Louis, MO). Cells were disrupted by

vortexing for 1 min. and cell debris removed by centrifugation at 3000g for 10 min. at 4°C. The resulting cell lysate supernatant was dispensed into small aliquots and stored at -85°C for quantification of intracellular viral content.

#### 2.4. Adenoviral vector titration

AdV titration was done in Anchorage-dependent 293 cells seeded into 6 well plates at  $1 \times 10^6$  cell/well. One day after, seeded cells were infected with 1 ml of serial dilutions of the AdV in MEM supplemented with 5% FBS. After 24 h of incubation the medium was removed and the cells covered with agarose mix: MEM 2× (Invitrogen, Glasgow, UK) supplemented with 1% (w/v) agarose (Sigma-Aldrich, St. Louis, MO), 5% (v/v) FBS and 15% (v/v) PBS. Plates were incubated in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C and observed at day 7 and day 14 for plaque forming units (pfu).

#### 2.5. Intracellular pH measurement

pH<sub>i</sub> was monitored in viable cell suspensions of approx.  $1 \times 10^6$  cell/ml. Briefly, samples collected from the culture bulk, were centrifuged at 200g for 10 min. at 4°C and the cell pellet resuspended in Earl's balanced salt solution (EBSS) buffer pH 7.3 (140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 5 mM glucose (all from Merck, Darmstadt, Germany) and 25 mM HEPES (Sigma-Aldrich, St. Louis, MO)) supplemented with 10 μM of the well known cytosolic pH dye BCECF-AM (Invitrogen, Glasgow, UK); Data was acquired after incubation of the cell suspension for 45 min. at 37 °C (to allow BCECF-AM to be hydrolysed by intracellular esterase into pH-dependent fluorescent BCECF).

The calibration curve was done using labelled cells resuspended in high [K<sup>+</sup>] buffer with pH of 6.0-8.0 in 0.2 pH units steps prepared by mixing appropriate proportions of 135 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM NaCl and 110 mM

K<sub>2</sub>HPO<sub>4</sub>, 20 mM NaCl (all from Merck, Darmstadt, Germany), containing 10 µM nigericin for 5 min. before flow cytometry.

Data was acquired using a Flow cytometer CyFlow® Space (Partec, Germany) equipped with a blue solid state laser set at 488 nm. The green fluorescence (FLI) was selected using a 525 nm band pass filter, while the red fluorescence was obtained with a 610 nm long-pass filter (FL3). The ratio of the two signals was used in all experiments, directly calculated by the cytometer data analysis system. The ratio is given by the mean peak channel of the histogram ratio after analysis of 2.5×10<sup>4</sup> cells. Data analysis was performed using FlowMax® software (Partec, Germany).

#### 2.6. DNase II activity assay at different pH values

For the DNase II activity assay, 5 ml of a cell suspension (3×10<sup>6</sup> cell/ml) in buffer containing 100 mM sodium acetate, 5 mM EDTA (Merck, Darmstadt, Germany) and 0.1% (v/v) Triton were disrupted by vortexing for 1 min. followed by centrifugation at 3000g for 10 min. at 4°C. Afterwards, 250 µl of AdV DNA was added to the supernatant; these were subsequently incubated at 37°C during 1h at a pH range of 5.6-7.8 in 0.2 pH units. This AdV DNA was obtained by incubation of 2.5×10<sup>7</sup> AdV (infectious particles) in 0.1% (v/v) SDS (Merck, Darmstadt, Germany) at 56 °C for 10 min.

#### 2.7. Determination of adenovirus DNA by quantitative real time PCR

For the quantitative real time PCR a plasmid containing one copy of the gene of interest, was used as external standard for quantification; the size of the amplicon is 220 bp. DNA amplifications were done using the Light Cycler system with “Fast Start Master SYBR Green I kit” (Roche Diagnostics, Germany). For a 20 µl PCR reaction, 2 µl DNA template and 18 µl of Mastermix were added to each capillary. The Mastermix was prepared to obtain a final concentration of 3 mM MgCl<sub>2</sub> and 0.5 µM of each primer in

each capillary. The oligonucleotide primers used were: forward primer (5'-TCG TAG AAG TGG ACG GT-3') and reverse primer (5'-GTG TTC GTG ACT GAA GC-3'). The primers were designed to blast only the gene of interest. AdV DNA was denatured for 10 min. at 95°C. The standard amplification program included 30 three-step cycles: (i) denaturation-heating at 95°C for 1 s, (ii) annealing-cooling at 56°C during 5 s, and (iii) elongation-heating at 72°C for 10 s. Fluorescence was acquired at the end of the elongation step of each cycle. Data were analysed using the Light Cycler software and the "fit points method" described elsewhere (Rasmussen et al., 1998).

## 2.8. Analytical Methods

Cell concentration was determined by counting cells on a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany). Cell viability was determined by incubation of 293 cells with 1 µg/ml propidium iodide (PI) (Invitrogen, Glasgow, UK). The cell suspensions were analysed in the flow cytometer - CyFlow® Space (Partec, Germany).

Ammonia was quantified enzymatically using a UV test number 1112732035 (Boehringer Mannheim, R-Biopharm AG, Germany).

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of ammonia addition on adenovirus vector production

To evaluate the effect of extracellular ammonia on cell specific productivity, NH<sub>4</sub>Cl was added to the culture medium at the time of infection at CCI of 1×10<sup>6</sup> cell/ml. The viruses were harvested at 48 hpi and titrated. The results obtained show that, for all the tested concentrations (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mM NH<sub>4</sub><sup>+</sup>), there is no significant effect on AdV production (Figure 3.2.2).

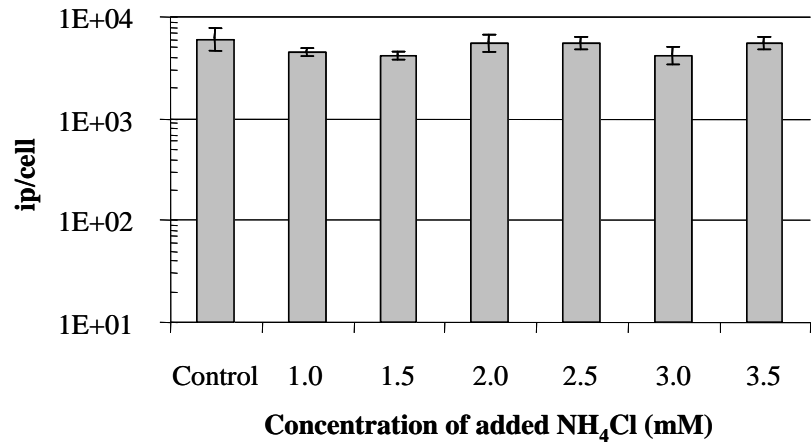


Figure 3.2.2. Effect of added ammonia on cell specific productivity. Cells were infected at a CCI of  $1 \times 10^6$  cell/ml with a MOI of 10 in non ammoniagenic medium after addition of NH<sub>4</sub>Cl and harvested at 48 hpi.

An inhibitory effect of extracellular ammonia on AdV production for concentrations above 1.5 mM has been reported (Ferreira et al., 2005b), this causing major problems for AdV production at high cell densities. However these studies were done without external addition of NH<sub>4</sub><sup>+</sup> to the culture medium, the inhibitory effect becoming a direct consequence of the accumulation of ammonia derived mainly from cell metabolism, i.e. deamination of glutamine. The results reported herein (figure 3.2.2) suggest that ammonia has a different role when derived from an intracellular origin. Thus, an experimental approach allowing to study the effect of increasing concentrations of produced ammonia will mimic more faithfully the environment that the cells face in the bioreactor during AdV production. This can be achieved using non-ammoniagenic media.

### 3.2. Effect of ammonia on intracellular pH during cell growth

To evaluate the effect of produced ammonia on pH<sub>i</sub> during cell growth, 293 cells adapted to grow in non-ammoniagenic medium ( $q_{\text{ammonia}} = 0.09 \times 10^{-9}$  mmol/cell.h) were compared with 293 cells growing in ammoniagenic

medium ( $q_{\text{ammonia}} = 4 \times 10^{-9}$  mmol/cell.h) in terms of growth, ammonia production and  $\text{pH}_i$ . As shown in Figure 3.2.3, for the non-ammoniagenic medium, the cells were able to maintain the  $\text{pH}_i$  between 7.2 and 7.3 throughout all the culture; for ammoniagenic medium the  $\text{pH}_i$  was considerably lower: a drop of 0.2 units was evident at time 0 h and, as cells reached the concentration of  $2 \times 10^6$  cell/ml (at 96 h), a significant decrease in  $\text{pH}_i$  was registered.

For the non-ammoniagenic medium, the  $\text{pH}_e$  was maintained at 7.20 during all the culture time. For the ammoniagenic medium, a decrease of 0.05 (from 7.20 to 7.15) was observed from time 0 to time 96; however, this decrease was not as significant as the one observed for the  $\text{pH}_i$ .

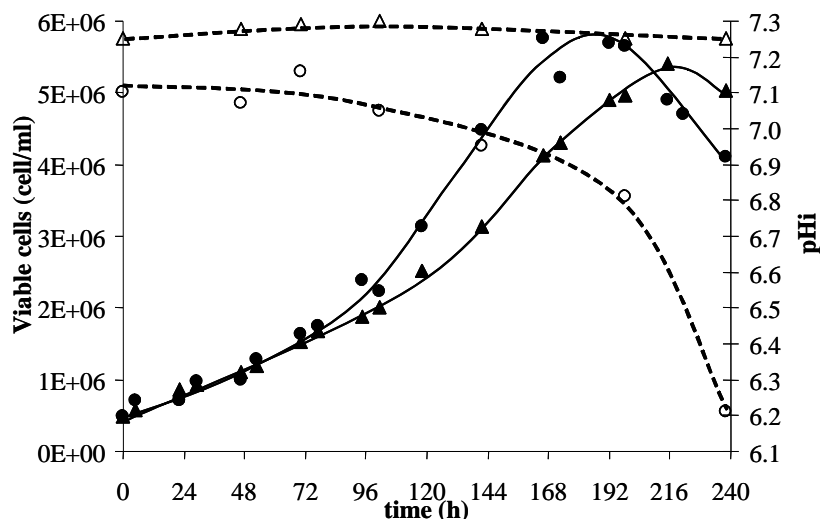


Figure 3.2.3. Evaluation of  $\text{pH}_i$  (empty symbols) during 293 cells growth (full symbols) in ammoniagenic medium (circles) and non-ammoniagenic medium (triangles).

By monitoring ammonia concentration it is possible to correlate the changes observed in  $\text{pH}_i$  with the ammonia production (Figure 3.2.4). As expected, for the non-ammoniagenic medium the production of ammonia



was significantly lower than for the ammoniagenic medium. The observed delay in pH<sub>i</sub> decrease relative to the faster enhancement of ammonia concentration in the culture medium can be related with the increasing number of “proton shuttle” cycles (McQueen and Bailey, 1990; Wu et al., 1993) leading to a much higher uptake of protons than the overall cell capacity to excrete them.

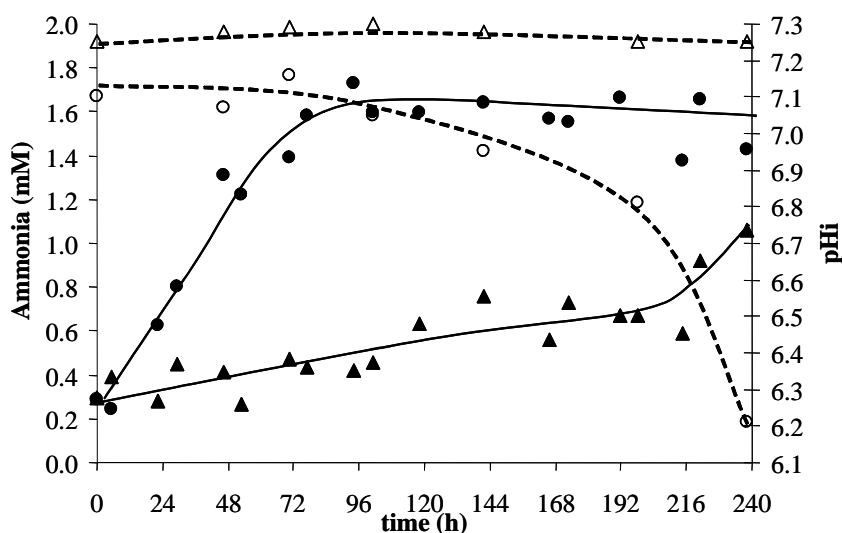


Figure 3.2.4. Relationship between pH<sub>i</sub> (empty symbols) and ammonia production (full symbols) in ammoniagenic medium (circles) and non-ammoniagenic medium (triangles).

### 3.3. Evaluation of ammonia production and intracellular pH during adenovirus vector production at different CCI

In order to evaluate the effect of produced ammonia on pH<sub>i</sub> during the production of AdV at a CCI of 1 and  $3 \times 10^6$  cell/ml, 293 cells were infected in either non-ammoniagenic and ammoniagenic media. As can be observed in Figure 3.2.5, the pH<sub>i</sub> is severely affected after infection, independently of the medium and CCI used. However, for both culture media tested, this

decrease is more pronounced when the infection is performed at CCI of  $3 \times 10^6$  cell/ml, especially for ammoniagenic media.

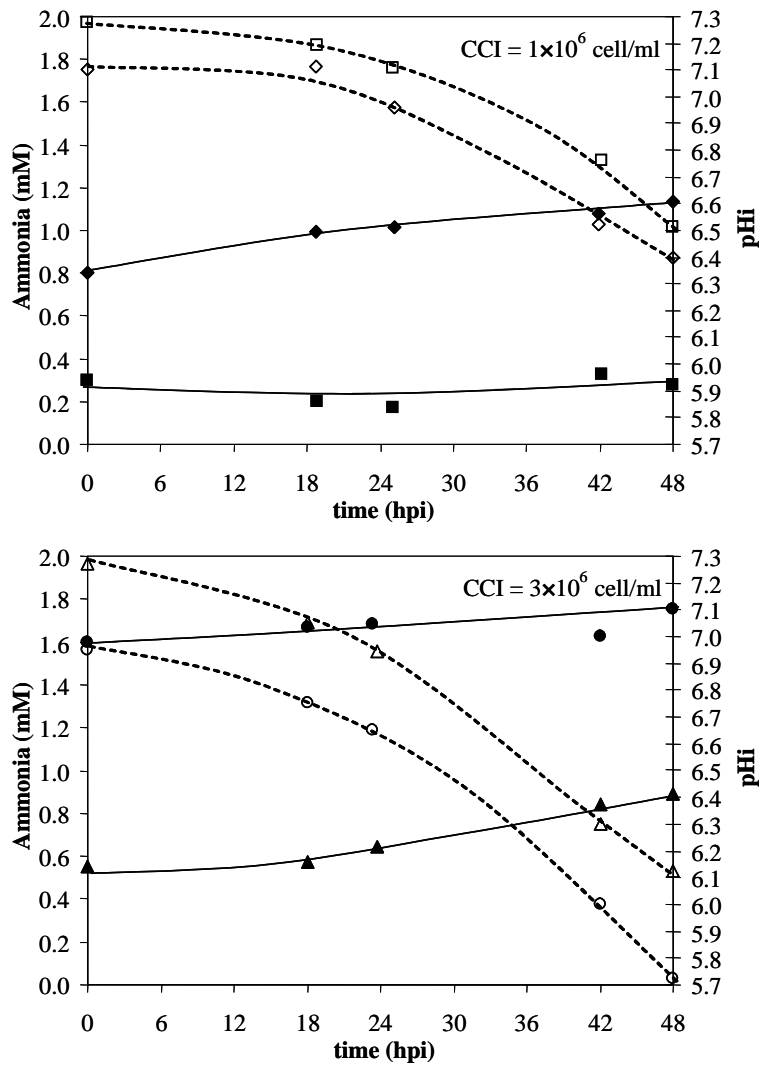


Figure 3.2.5. Evaluation of pH<sub>i</sub> (empty symbols) and ammonia (full symbols) during AdV productions at CCI of  $1 \times 10^6$  cell/ml in ammoniagenic medium (squares) and non-ammoniagenic medium (diamonds) and CCI of  $3 \times 10^6$  cell/ml in ammoniagenic medium (circles) and non-ammoniagenic medium (triangles) with a MOI of 10.

The specific ammonia production rate was not significantly affected when the CCI increased from 1 to  $3 \times 10^6$  cell/ml, thus the observed pH<sub>i</sub> decrease (Figure 3.2.5) seems to be promoted by other factors than the ammonia production. Matsuyama et al. (2000) shown that a very early event in apoptosis involves a change on the pH<sub>i</sub> regulation, meaning that the significant decrease observed in pH<sub>i</sub> after AdV infection may be a way to the cell respond to infection by entering in apoptosis. In addition, Matsuyama et al. (2000) have shown that the efficiency of caspase activation, responsible for the beginning of the apoptosis process, is pH sensitive, with an in vitro pH optimum of approximately 6.6. In fact, the amount of viable cells after infection at CCI of  $3 \times 10^6$  cell/ml start to undergo a more pronounced decrease when the pH<sub>i</sub> reaches the value of 6.6 (Figure 3.2.5) at 24 hpi for the ammoniagenic medium versus 36 hpi for the non-ammoniagenic medium (Figure 3.2.6); this means that cells are kept viable after infection for a longer time when the non-ammoniagenic medium is used.

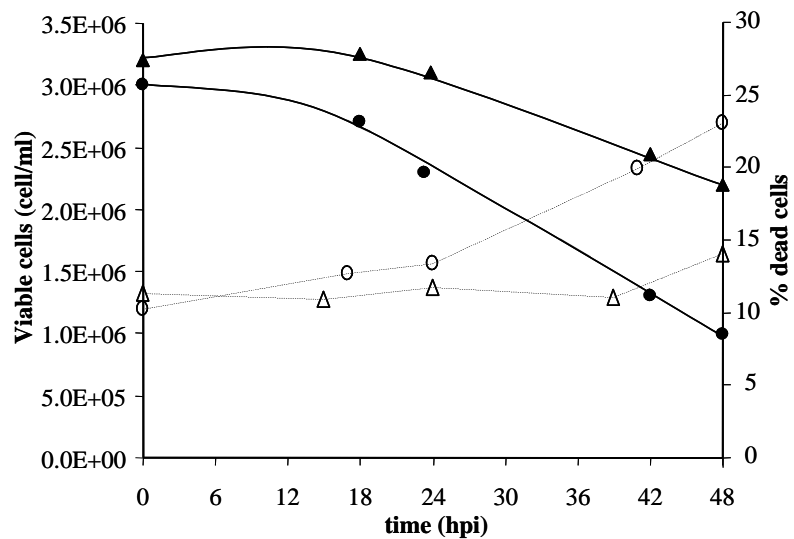


Figure 3.2.6. Effect of CCI of  $3 \times 10^6$  cell/ml in cell viability (full symbols) and percentage of dead cells (empty symbols) in ammoniagenic medium (circles) and

non-ammoniogenic medium (triangles).

### 3.4. DNase II effect upon adenovirus vector DNA at different pHs

To evaluate how pH<sub>i</sub> affects DNase II activity and consequently AdV DNA, incubations of the viral DNA were done at different pH values. As shown in Figure 3.2.7, pH affects the activity of DNase II upon the AdV DNA, a significant decline being observed for pH values below 6.6. No significant effects in AdV DNA were observed for similar studies performed with DNase I (data not shown).

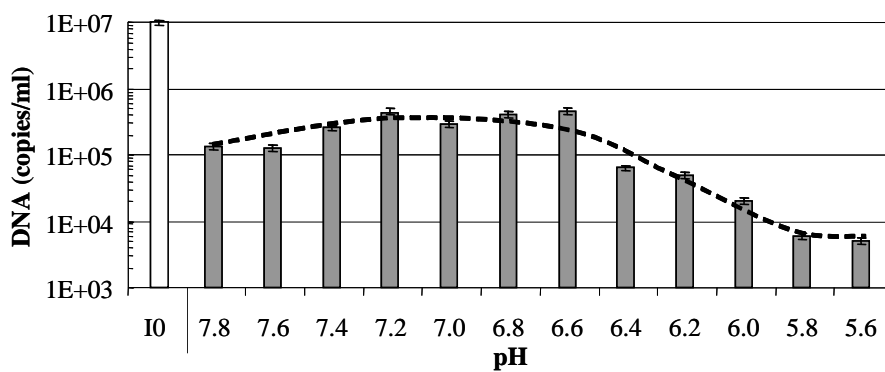


Figure 3.2.7. Effect of pH on the activity of DNase II. AdV DNA was incubated with a cell supernatant extract as described in material and methods. (□) Represents the initial amount of AdV DNA observed at time 0 for each pH tested.

Comparing the activity of DNase II with the pH<sub>i</sub> profiles after infection at a CCI of 1 and  $3 \times 10^6$  cell/ml in the ammoniogenic and non-ammoniogenic medium (Figure 3.2.5), it is possible to conclude that at CCI of  $1 \times 10^6$  cell/ml, independently of the medium used, only at the end of the production process the pH<sub>i</sub> reach values below 6.6. On the other hand, when cells are infected at CCI of  $3 \times 10^6$  cell/ml in the ammoniogenic medium, the pH<sub>i</sub> reach the 6.6 units faster than in the non-ammoniogenic

medium. Thus, for ammoniagenic media, due to the significant increase on DNase II activity for values below pH 6.6, more AdV DNA degradation will occur leading to less availability of virus DNA for encapsidation with a consequent decrease in bioactive AdV production.

### 3.5. Effect of intracellular pH on adenovirus vector production

To elucidate the  $pH_i$  effect upon AdV production, 293 cells adapted to grow in the non-ammoniagenic medium were infected at a CCI of  $1 \times 10^6$  cell/ml at pH 7.0 or at pH 7.3 ( $pH_i$  observed at the time of infection at CCI of  $3 \times 10^6$  cell/ml in the ammoniagenic and non-ammoniagenic media respectively), with or without nigericin addition and harvested at 48 hpi. Nigericin was used to mimic the  $pH_i$  observed at CCI  $3 \times 10^6$  cell/ml in both media at  $pH_i$  7.0 and 7.3.

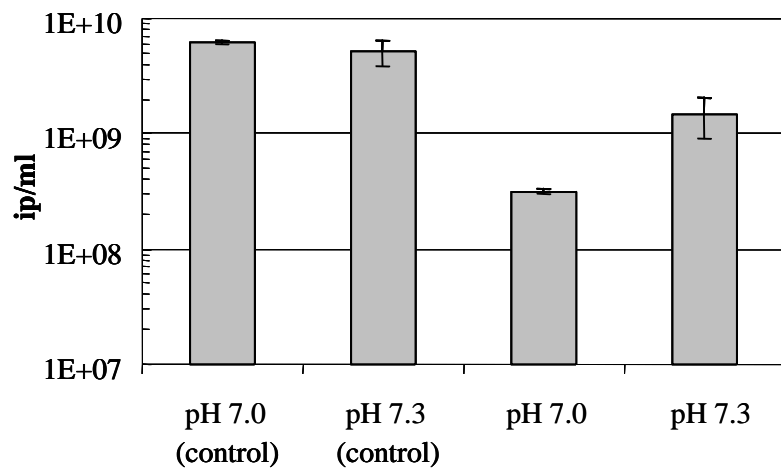


Figure 3.2.8. Effect of  $pH_i$  on AdV volumetric productivity.  $pH_i$  was decreased by addition of 30 mM KCl and of 50  $\mu$ M nigericin as described in material and methods.

As can be observed in Figure 3.2.8, although there is no significant difference on AdV production at pH 7.0 or at pH 7.3 in control conditions,

when nigericin is used a drop in AdV volumetric productivity is observed for both pHs, although it is more pronounced at pH 7.0. Despite the fact that nigericin has a residual cytotoxic effect (data not shown), it is obvious that, for a pH of 7.0, AdV volumetric productivity is strongly affected.

These results reflect the impact of the  $\text{pH}_i$  on AdV production and the overall need to minimize all the factors that contribute to its decrease, such as the intracellular ammonia production.

#### 4. CONCLUSIONS

The present study compares the effect of two different culture media leading to different specific ammonia production rates, upon  $\text{pH}_i$  and AdV productivity in 293 cells infected at different CCI<sub>s</sub> ( $1$  and  $3 \times 10^6$  cell/ml). During cell growth the  $\text{pH}_i$  decreased significantly in the ammoniagenic medium, where the specific ammonia production rate is higher; furthermore, after infection, the  $\text{pH}_i$  dropped significantly at both CCI<sub>s</sub> for both media, although the lowest  $\text{pH}_i$  was obtained for the ammoniagenic medium. These changes observed in the  $\text{pH}_i$  were found to play an important role on both processes of apoptosis and AdV DNA degradation, leading to a decrease in bioactive AdV production, more severe for the ammoniagenic medium.

The data reported herein identifies  $\text{pH}_i$  as one of the key factors responsible for the “cell density effect” observed on AdV production. Non-ammoniagenic medium was shown as a good option to be implemented at the bioreaction level that will lead to the maintenance of cell viability during AdV production and consequent increase on cell specific productivity.

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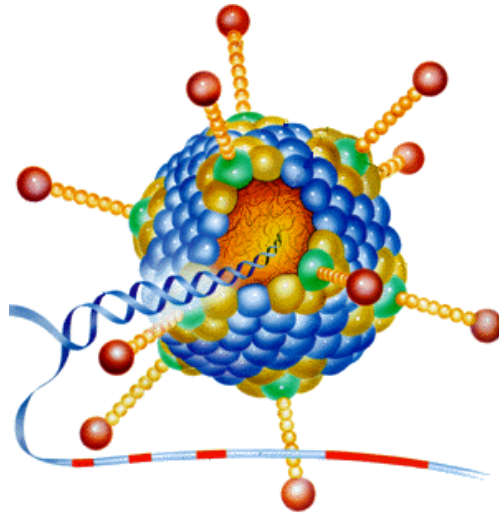
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## CHAPTER IV

### Cell Cycle



## PART 1

The importance of 293 cell cycle phase on adenovirus vector production

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## Abstract

One of the major restrictions on the production of adenoviral vectors (AdV) is the so-called “cell density effect”, i.e., a drop in cell specific productivity concomitant with increased cell concentration at infection (CCI) above  $1 \times 10^6$  cell/ml. Although this “cell density effect” has been considered by several authors as a consequence of nutrient depletion and/or accumulation of byproducts, this study shows that the cell fraction at the S phase of the cell cycle may also play a role on AdV production at high CCI in 293 batch cultures. By infecting cells obtained from different growth stages at cell densities of 0.7 (lag), 1.6 (middle exponential), 3.3 (late exponential) and  $5.0 \times 10^6$  (stationary) cell/ml, consisting of a descending proportion of cells in the S phase in the cell cycle in equalized cell density condition, it is shown that cell specific productivity is dependent on the cell cycle phase at infection at both the optimal CCI ( $1 \times 10^6$  cell/ml) and high CCI ( $4 \times 10^6$  cell/ml). In particular, under the equalized cell density conditions, the infection of cells with an S phase proportion of 50% at the high CCI gave the higher AdV volumetric productivity. Moreover, the lowest cell specific productivity was obtained with the lowest S phase proportion of 28% for both CCIs. These results indicate that a decrease in the proportion of cells at S phase at the time of infection also contributes to a decreased cell specific productivity at high cell densities.

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## 1. INTRODUCTION

AdV have been extensively used as vectors for both gene therapy and recombinant DNA vaccines, which have been tested in both humans and different animal species (Ferreira et al., 2005a). The need for large quantities of clinical-grade AdV is an important limitation to in vitro experimentations as well as to pre-clinical and clinical studies (Nadeau and Kamen 2003; Kamen and Henry 2004). This limitation is due to the fact that the cell specific productivity decreases sharply with increased CCI above  $1 \times 10^6$  cell/ml, the so called “cell density effect”. So far, this “cell density effect” has been attributed to nutrient limitations and/or accumulation of byproducts (Nadeau and Kamen 2003; Henry et al., 2004; Kamen and Henry 2004; Ferreira et al., 2005b). Although metabolic flux analysis has been used to characterize the effect of various environmental conditions upon cell metabolism, the exact nature of the factors limiting AdV productivities at high cell density remains unknown (Nadeau et al., 2000a; Nadeau et al., 2000b; Nadeau et al., 2002). Therefore, present strategies for overcoming this bottleneck have been focused on the improvement of cell growth environment during and after infection. These strategies include medium replacement at the time of infection and the addition of glucose at 24 hour post infection (hpi) together with periodical pH adjustments, allowing a sustained maximum specific productivity at  $1.6 \times 10^6$  cell/ml (Garnier et al., 1994). This strategy, associated with essential amino acids feeding at 24 hpi, is able to stabilise the volumetric productivity at cell densities up to  $2 \times 10^6$  cell/ml (Nadeau et al., 1996). Perfusion mode operation has been demonstrated as the most effective measure to control the culture environment and to remove toxic byproducts (Cortin et al., 2004; Henry et al., 2004; Kamen and Henry 2004). Nevertheless, the cell specific productivity could only be maintained by infecting cells at densities up to  $3 \times 10^6$  cell/ml using high perfusion rate at 2 reactor volumes per day (VVD), at 2 days post-infection, a very costly



proposition (Henry et al., 2004; Kamen and Henry 2004). By decreasing the temperature to 35 °C post infection, Cortin et al. (2004) have further increased CCI up to  $8 \times 10^6$  cell/ml at perfusion rate of 1 VVD with a production of  $7.8 \times 10^9$  infectious particles/ml (ip/ml). In studies conducted by the authors under much less expensive fed batch mode using 293 cells adapted to non-ammoniagenic medium an 1.8 fold increase on AdV volumetric productivity at CCI  $3 \times 10^6$  cell/ml was obtained, when comparing with infection at CCI  $1 \times 10^6$  cell/ml in the same medium, with a production of  $8.5 \times 10^9$  ip/ml (Ferreira et al., 2005b; Ferreira et al., 2005c).

During cell culture processes, especially in batch and fed-batch mode, apart from the continuously changing culture chemical and physical environment, the cells themselves are also continuously varying their physiological and metabolic states. It is well known that during the culturing, the population of cells goes through four growth stages, i.e., lag, exponential, stationary and death-phase, where an individual cell commits to four successive growth phases in the cell cycle, i.e., G1 (gap one phase, between mitosis and DNA synthesis), S (DNA synthesis phase), G2 (gap two phase, between completion of DNA synthesis and mitosis) and M-phase (mitosis phase). In some cases, cells may escape from the cell cycle and enter a resting phase (G0), from where, given suitable condition, they can return to the cell cycle. During the culture process, the cell cycle phase distribution may be continuously changing and no matter in which phase of the cell cycle the cells are infected by AdV, the viral DNA synthesis will only take place 9h after infection (Hodge and Scharff 1969).

The effect of cell cycle phase at the time of infection on AdV productivity has not been well described. The scarce reported data suggests that it is both cell line and virus type dependent. For avian AdV, both production of infectious virus and viral DNA synthesis are correlated with events during the S phase of the infected chicken embryo fibroblasts cells (Kraft and Tischer 1978). For HeLa cells, infection with human Ad5 during S phase produced greater yields of E1B 55-kDa-mutant and E4 orf6-mutant viruses

than did cells infected during G1 or asynchronous cells. However the production of wild Ad and E4 orf3-mutant Ad was not significantly restricted by the cell cycle (Goodrum and Ornelles 1997). In addition, for many other viruses, the production is associated with the S phase of the cell cycle. For example, coxsackievirus production is dependent on G1 or G1/S phase (Feuer et al., 2002); for minute virus of mice, a parvovirus, the conversion of the viral genome from input single-stranded DNA to a double-stranded DNA, the form which undergoes further replication, appears to be an S phase-specific event (Wolter et al., 1980); in the baculovirus-insect cell expression system, the infection yield at G1 or S phase-infection was 1.5-1.8-fold higher than that at G2/M phase-infection (Saito et al., 2002); the replication of the bovine herpesvirus-4 depends on transition through S phase (Vanderplasschen et al., 1995); the herpes simplex virus type 1 vmw65 (VP16) insertion mutant depends on cells infected during S phase for early protein synthesis and replication (Daksis and Preston 1992).

Thus the aim of this work is to investigate the importance of the cell cycle phase of the host 293 cells at the time of infection upon AdV productivity, so that more efficient infections at high cell density may be achieved.

## 2. MATERIALS AND METHODS

### 2.1. Cell line and culture maintenance

Suspension-adapted 293 cells (ATCC-CRL-1573, Rockville, MD) were grown in shake flasks (Corning, NY) in commercially available and chemically defined medium without serum and protein, CD293, supplemented with 4 mM of L-glutamine (all from, Invitrogen, Glasgow, UK) at 160 rpm under an humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C. The cells were routinely propagated twice a week with an inoculum density of  $0.5 \times 10^6$  cell/ml.

## 2.2. Cell infection

A replication-defective AdV derived from type 5 AdV was kindly provided by Dr. Tom Barret (IAH-Pirbright, UK). Infection was carried out in 125 ml shake flasks with working volumes of 40 ml using a multiplicity of infection (MOI) of 10 (see Annex A). Infected 293 cells were harvested at 0, 24, 48 and 72 hpi and prepared as previously described in Ferreira et al. (2005b)

## 2.3. Analytical methods

Cell concentration and viability were determined by counting cells on a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany) using the trypan blue (Invitrogen, Glasgow, UK) dye exclusion method.

AdV titration was performed by the end-point dilution method (TCID<sub>50</sub>) using 96 well plates and anchorage-dependent 293 cells cultured in T-flasks (Sarstedt); these cells were cultured in MEM supplemented with heat-inactivated 5% fetal bovine serum (all from Invitrogen, Glasgow, UK) and 2 mM L-glutamine under an humidified atmosphere of 5% CO<sub>2</sub> in air at 37 °C. The titer was determined according to the method of Spearman and Kraber, as described elsewhere (Darling et al., 1998).

Glucose and lactate were analyzed using an YSI Multiparameter Bioanalytical System Model 7100 MBS (Yellow Springs, USA).

Ammonia was quantified enzymatically using the UV test number 1112732035 (Roche Diagnostics GmbH, Mannheim, Germany).

## 2.4. Flow cytometric analysis

To quantify the cell-cycle distribution, cells were stained with propidium iodide to measure the DNA content by flow-cytometric analysis (FACS). Cells were centrifuged at 200 g for 10 min at 4°C, washed with ice-cold PBS

and stained with ice-cold Vindelov's solution (Vindelov et al., 1983): 1 g/l trisodic citrate, pH 7.6 (Sigma); 50 mg/l propidium iodide (Sigma); 0.1% NP 40 (Roche); 700 U/l RNase A (Sigma); 0.01 M NaCl (Merck) for a final cell concentration of  $1.5 \times 10^6$  cell/ml. All FACS analyses were performed on a FACSCalibur™ using the Cell Quest™ software (Beckton-Dickinson, San Jose, CA).

### 3. RESULTS AND DISCUSSION

#### 3.1. Relationship between cell density and cell cycle phase distribution during normal batch cultures of 293 cells

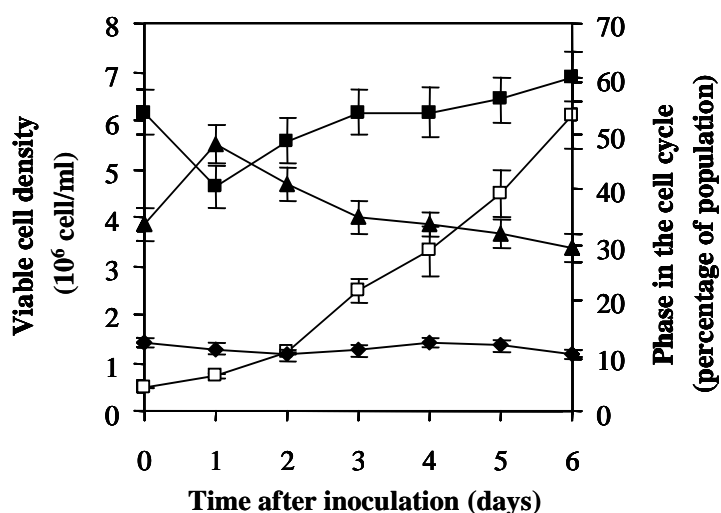


Figure 4.1.1. Profiles of cell growth and cell cycle phase distribution during 293 cell suspension batch cultures. Symbols:  $\square$ , cell density;  $\diamond$ , G2/M phase;  $\blacksquare$ , G1/G0 phase;  $\blacktriangle$ , S phase.

Figure 4.1.1 describes the cell cycle phase distribution at different cell densities during a normal batch cultivation process of 293 cells. After inoculation, the proportion of cells in the S phase of the cell cycle

increased sharply, with a 70% increase within the first 24 h, from ca. 30% to ca. 50% of the total cell population. Conversely, the percentage of G1/G0 cells in the cell culture fell during this period. The highest S phase proportion occurred at the middle of the lag phase of the growth stage, corresponding to a cell density of  $0.6\text{--}0.8 \times 10^6$  cell/ml. Thereafter, the percentage of cells in the S phase decreased gradually, as the cell density increased above  $1 \times 10^6$  cell/ml; correspondingly, the percentage of G1/G0 cells in the cell culture rose, while the percentage of cells at G2/M phase was kept relatively stable at about 11% during the whole growth cycle. Accompanying the cell density increase, the nutrients in the culture medium, namely glucose and glutamine, decreased quickly; at the same time, the accumulation of the byproducts, specially lactate and ammonia, increased rapidly in the 293 cell suspension batch cultures (Figure 4.1.2).

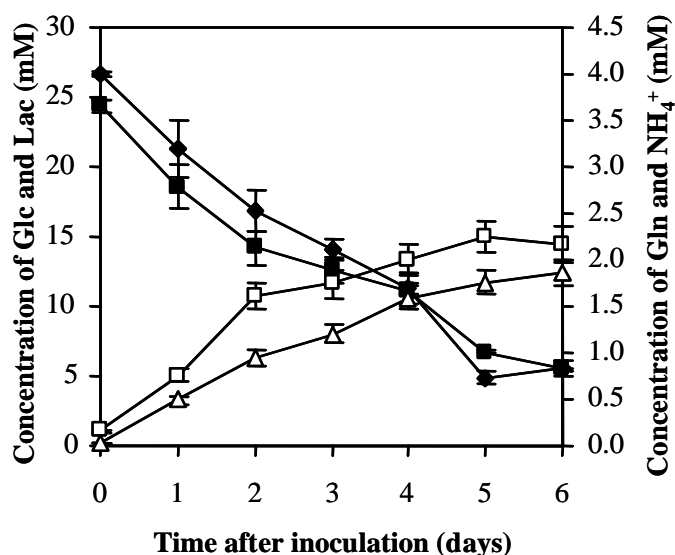


Figure 4.1.2. Kinetics of total glucose (Glc, ■) and glutamine (Gln, ♦) consumption and total lactate (Lac, □) and ammonia (NH<sub>4</sub><sup>+</sup>, Δ) production of 293 cell suspension batch cultures.

Although Lullau et al. (2003) observed for 293-BACE-Fc a higher S phase

fraction at the end of batch culture, the trend in the cell cycle phase distribution of 293 cells obtained here is well supported by others who observed similar results for cultures of hybridoma cells (Ramirez and Mutharasan 1992; Balcarcel and Stephanopoulos 2001; Luo and Yang 2004), CHO cells (Ley and Tobey 1970; Tobey and Ley 1970) and Hela cells (Koza and Herbst 1992; Goodrum and Ornelles 1997).

### 3.2. Effect of different proportion of cells under S phase in equalized cell density conditions upon AdV productivity

As demonstrated above, the proportion of cells in the S phase decreased as the cell density rose. To evaluate if this decreased proportion of cells in the S phase at high cell density correlates with the so called “cell density effect”, cells were infected at different growth stages with different proportions at S phase under equalized cell density conditions at CCI of  $1 \times 10^6$  cell/ml. In brief, after harvesting, the cells were centrifuged to totally remove the spent medium, and then resuspended in fresh medium at a final concentration of  $1 \times 10^6$  cell/ml before infection, and infected with the same MOI at the same time. As stated in the Introduction, the CCI of  $1 \times 10^6$  cell/ml was chosen on the basis of the observations that it is the optimum CCI for normal AdV production (Ferreira et al., 2005c). The medium exchange was performed to avoid lack of nutrients and presence of toxic byproduct thus permitting to isolate the evaluation of the cell cycle effect. The results are illustrated in Figure 4.1.3 and summarized in Table 4.1.1.

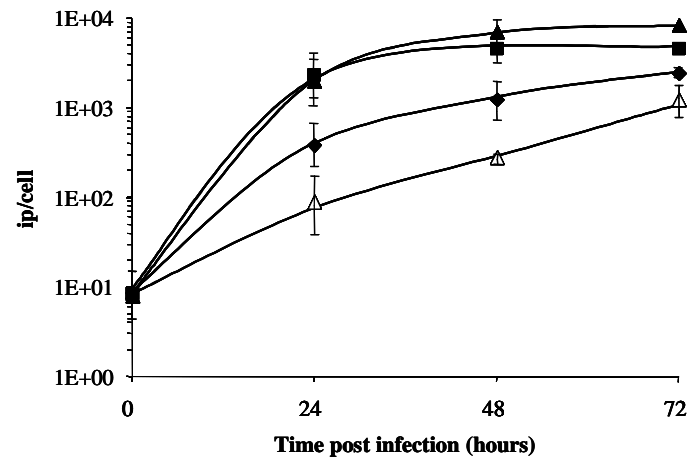


Figure 4.1.3. Profiles of cell specific productivity of cells obtained from different growth stages with different proportions in S phase at the time of infection in equalized cell density conditions at a CCI of  $1 \times 10^6$  cell/ml. The 293 cells for this experiment were harvested from lag, middle exponential, late exponential and stationary phases, corresponding to cell densities of 0.7, 1.6, 3.3, and  $5 \times 10^6$  cell/ml, with proportions of cells in S phase of 50% (▲), 40% (■), 32% (◆) and 28% (Δ), respectively. The cells were centrifuged to discard the spent medium and replaced with fresh medium at CCI of  $1 \times 10^6$  cell/ml, in order to provide an equalized infection environmental condition for all the cells.

Table 4.1.1. Effect of cells obtained from different growth stages with different proportions in S phase at the time of infection in cell specific productivity at the optimal cell concentration at infection ( $1 \times 10^6$  cell/ml). Infections were performed at MOI of 10 and AdV harvested at 72 hpi.

Growth stage of cells	Original cell density (cell/ml)	Percentage of S phase cells at infection	Maximum cell specific productivity (ip/cell)
Lag	$0.7 \times 10^6$	$50\% \pm 3\%$	$8.3 \times 10^3 \pm 0.7 \times 10^3$
Middle exponential	$1.6 \times 10^6$	$40\% \pm 3\%$	$4.7 \times 10^3 \pm 0.4 \times 10^3$
Later exponential	$3.3 \times 10^6$	$32\% \pm 2\%$	$2.5 \times 10^3 \pm 0.3 \times 10^3$
Stationary	5.0	$28\% \pm 2\%$	$1.3 \times 10^3 \pm 0.5 \times 10^3$

The infection of cells from the lag phase, with the highest proportion at S phase, resulted in the highest productivity of  $8.3 \times 10^3$  ip/cell at 72 hpi, 6.4 times higher than the level of cells obtained from stationary phase with the lowest proportion in S phase at the same time ( $1.3 \times 10^3$  ip/cell). In other words, even though all infections were performed at the optimal CCI ( $1 \times 10^6$  cell/ml), cells obtained from different growth stages exhibited different cell specific productivities. These results strongly support the hypothesis that the cell specific productivity is dependent upon cell cycle at infection, i.e., higher proportion of cells at S phase, corresponding to a larger cell population under faster division, resulted in higher AdV productivities. Interestingly, cells obtained from the growth stages at higher cell densities of  $3.3$  and  $5.0 \times 10^6$  cell/ml presented a lower cell specific production rate and a higher optimal harvesting time of 72 hpi although 48 hpi is the optimal harvesting time for the cells obtained from the growth stages at lower cell densities of  $0.7$  and  $1.6 \times 10^6$  cell/ml (Figure 4.1.3). Although this effect could be correlated with cell aggregation in the experiments performed at high cell densities, no significant cell aggregation (no more than 2-4 cells aggregates) was found. Moreover, an increase of 60% on viable cell density at 24 hpi for cells with the higher proportion in S phase was observed (Figure 4.1.4). Hodge and Scharff (1969) observed that no matter in which phase of the cell cycle the cells are infected, the viral DNA will start to be produced at 9 hpi, meaning that a cell in G1 phase will stop at the S phase and that a cell in the S phase will double once more after infection; therefore it is not surprising to observe cell growth after infection where the proportion of cells on the S phase of the cell cycle is higher.



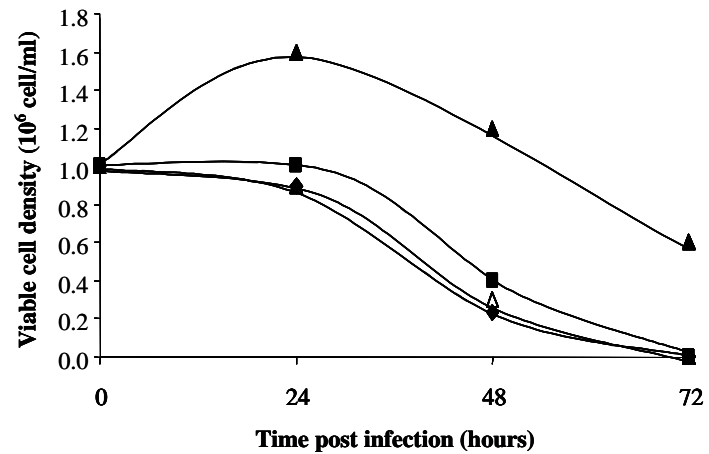


Figure 4.1.4. Profiles of cell growth after infection in equalized cell density conditions at a CCI of  $1 \times 10^6$  cell/ml. The 293 cells for this experiment were harvested from lag, middle exponential, late exponential and stationary phases, corresponding to cell densities of 0.7, 1.6, 3.3, and  $5 \times 10^6$  cell/ml, with proportions of cells in S phase of 50% (▲), 40% (■), 32% (◆) and 28% (Δ), respectively.

The effect of different proportion of cells under S phase at infection on AdV productivity was further investigated at higher CCI in the equalized cell density conditions. In this case, the CCI of  $4 \times 10^6$  cell/ml was chosen in order to highlight the difference in cell specific productivity in the equalized cell density condition. As illustrated in Figure 4.1.5, similarly to the results achieved at the optimal CCI of  $1 \times 10^6$  cell/ml, at the CCI of  $4 \times 10^6$  cell/ml the infection of cell cultures with higher proportion in S phase also resulted in higher cell specific productivity. At this CCI, the infection of cells with the highest S phase proportion (50%), resulted in a production of  $2.5 \times 10^3$  ip/cell at 72 hpi. This cell specific productivity was 16.7-fold higher than that obtained from infection of cells from the stationary growth stage at a cell density of  $5 \times 10^6$  cell/ml, consisting of the lowest S phase cell proportion (28%), and resulted in the higher volumetric productivity of  $1 \times 10^{10}$  ip/ml (Figure 4.1.5). Once more, this constitute strong evidence that the proportion of cells in S phase at infection is an

important parameter affecting cell specific productivity at both the optimum and even at higher CCI. However, for the same cell population, infections at CCI of  $1 \times 10^6$  cell/ml always resulted in a significantly higher cell specific productivity than infections at CCI of  $4 \times 10^6$  cell/ml; this may be due to the higher accumulation of ammonia and lactate at CCI of  $4 \times 10^6$  cell/ml (data not shown), excess of ammonia being inhibitory of AdV production (Ferreira et al., 2005b). In fact, concomitantly with lower cell specific productivity, the cell viability after infection dropped faster at CCI of  $4 \times 10^6$  cell/ml than it did at CCI of  $1 \times 10^6$  cell/ml (data not shown).

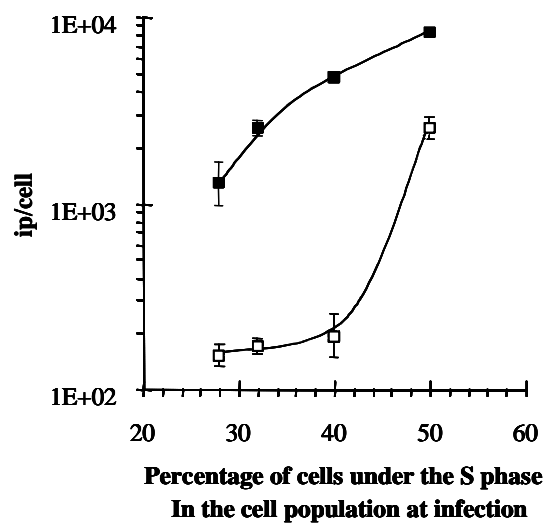


Figure 4.1.5. Comparison of maximal cell specific productivity obtained after infection at the optimal CCI of  $1 \times 10^6$  cell/ml (■) and at the high CCI of  $4 \times 10^6$  cell/ml (□) against proportion of cells in S phase at infection. The 293 cells for this experiment were harvested from lag, middle exponential, late exponential and stable phases, respectively, consisting of different proportion of cells in S phase (see text). Infections were performed at an MOI 10 and AdV harvested at 72 hpi.

As shown in this work, the AdV productivity is dependent upon the cell cycle phase at infection. Thus, cell specific productivity may be improved, especially at high CCI, by synchronizing 293 cells and infecting the cells at

higher proportions of cells in the S phase. However, the mechanism involved in this process is still unclear. It may be possible that the transcription and translation level of the E1 gene in the S phase of the 293 cells is higher than in G1/G0 phase. In addition, the larger volume of the cells in S phase than in G1/G0 phase may also be responsible for the higher cell specific productivity.

#### 4. CONCLUSION

This study shows that the proportion of cells at the S phase in the cell cycle decreased gradually at cell densities above  $1 \times 10^6$  cell/ml in 293 batch cultures. In addition, infections of cells from “naturally grown” populations at different densities with different proportions of cells in S phase in equalized cell density conditions demonstrate that cell specific productivity is cell cycle phase dependent at the time of infection. In particular, infection of cells obtained from lag growth phase, having the highest proportion of cells in S phase (50%) in the equalized condition, resulted in the higher AdV volumetric productivity of  $1 \times 10^{10}$  ip/ml at a CCI of  $4 \times 10^6$  cell/ml. These results indicate that the increase in the proportion of cells at the S phase in the cell population represents an increase on cell specific productivity at high cell densities. In addition, these results show also that the inoculum play a significant role, not only in the maximal cell density and specific growth rate [30], but also in cell specific productivity, being an important parameter to consider on AdV production.

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## PART 2

### 293 cell cycle synchronisation in adenovirus production

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## Abstract

As the market requirements for adenovirus vectors (AdV) increase, the maximisation of the virus productivity per culture volume per unit time is a key requirement. However, despite the fact that 293 cells can grow up to  $8 \times 10^6$  cell/ml in simple batch mode operations, for optimal AdV infection a maximum cell density of  $1 \times 10^6$  cell/ml at infection time has been reported. In addition, AdV production appears to be dependent of the cell cycle phase at the time of infection. To evaluate the dependence of AdV production on cell cycle phase, 293 cells were chemically synchronised at each phase of the cell cycle; a 2.6 fold increase on cell specific productivity was obtained when the percentage of cells at the S phase of the cell cycle was increased from 36% to 47%. A mathematical equation was used to relate AdV productivities with S phase cell synchronisation using this data.

A temperature shift strategy was also used attempted for synchronisation at the S phase, avoiding the use of chemical inhibitors. S phase synchronisation was possible by decreasing the culture temperature to 31°C during 67h and restoring it to 37°C during 72h. By using this strategy we were able to synchronise 57% of the population in the S phase of the cell cycle obtaining an increase of 7.3 fold on cell specific productivity after infection.

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## 1. INTRODUCTION

Adenoviruses are leading vectors for gene transfer, with products in development for applications ranging from cancer therapeutics to prophylactic vaccines and replacement therapies for genetic deficiencies (for reviews see Gallo et al. (2005), Ferreira et al. (2005a) and Morsy et al., (1998)); worldwide, over 300 clinical trials using Adenovirus Vectors (AdV) have already been approved, ongoing or completed, making AdV the most used delivery vector in clinical trials ([www.wiley.co.uk/genetherapy/clinical](http://www.wiley.co.uk/genetherapy/clinical)).

One of the goals of a commercial AdV cultivation process is to maximize the virus productivity per culture volume per unit time. The cell density at infection is a very important parameter as it impacts on the AdV volumetric productivity. However, despite the fact that 293 cells can grow up to  $8 \times 10^6$  cell/ml in simple batch mode operations, a maximum infection cell density of  $1 \times 10^6$  cell/ml has been reported as optimal (Nadeau and Kamen, 2003; Xie et al., 2003; Kamen and Henry, 2004; Ferreira et al., 2005c; Maranga et al., 2005). An approach to maintain cell specific productivity at increased cell density consists of infecting the cells after medium exchange (Garnier et al., 1994; Iyer et al., 1999; Ferreira et al., 2005b). However, this procedure adds the extra complexity of a cell separation step and increases in product production cost due to medium exchange. Other approach consists in the use of a fed-batch strategy, but its success has been limited (Nadeau et al., 1996; Ferreira et al., 2005b). By using a non-ammoniagenic medium an increase of 5 fold on cell specific productivity could be obtained at a CCI of  $3 \times 10^6$  cell/ml still bellow the expected (Ferreira et al., 2005b).

AdV production is usually performed assuming that cultured cells represent a uniform target for the virus. However, in fact, cells in culture are not homogenous, including a collection of cells with distinct physiologies. A major determinant of cell physiology in culture is the cell cycle with its

four stages (Stein et al., 1999) including elevated protein synthesis (G1 and G2 phases), elevated DNA synthesis (S phase) and changes in membrane trafficking, cytoskeletal organization and cell-cell interactions (M phase). In Chapter IV, Part 1 was shown that AdV production seems to be dependent of the cell cycle phase at the time of infection: during cell growth the highest percentage of cells at the S phase of the cell cycle is present at approximately  $1 \times 10^6$  cell/ml, which has been shown to be the optimal concentration for AdV infection, decreasing with increasing cell density (Zhang et al., 2006). Furthermore, Goodrum and Ornelles (1997) observed that in synchronously growing HeLa cells, approximately 75% of the cells infected during S phase, produced E1B AdV mutant virus, whereas only 10% of the cells infected during G1 phase were able to produce virus. Therefore, it is pertinent to synchronize cells at each phase of the cell cycle in order to identify the best phase for infection to increase cell specific productivity at high cell densities. Several methods for obtaining synchronised populations of mammalian cells in vitro have been reported by using genetic, chemical, environmental and physical strategies (Davis et al., 2001). For reversible chemical synchronisation several compounds are available, as thymidine (Law et al., 2006) for G1 phase synchronisation, mimosine (Hughes and Cook, 1996) for late G1 phase synchronisation, aphidicolin (Jackson, 1995), rapamycin (Law et al., 2006) and hydroxyurea (Rosenkranz and Becker, 1973) for synchronisation at the G1/S transition phase, staurosporine (Kocher and Clemetson, 1991) for G2 phase synchronisation and nocodazole (Ho et al., 2001) for synchronisation at the G2/M transition phase. However, in addition to the difficulty in ensuring their elimination in the final product, the use of these chemicals as synchronising agents also affects cell viability, often presenting severe limitations for large scale production purposes.

Besides chemical synchronisation, several studies suggest hypothermia as a potentially useful tool to manipulate mammalian cell cycle distribution (Rao and Engelberg, 1965; Siskin et al., 1965; Watanabe and Okada, 1967).

Typically, mammalian cells are grown at 37°C, mimicking mammalian body temperature. At “sub-optimal” temperatures (25-33°C) there is a prolongation of the cell cycle duration as a result of the temporal dilation of the G1, S and M (mitosis) phases, with G2 being the least and M the most sensitive phases to temperature shift (Rieder and Cole, 2002), undergoing the major increase in duration (Rao and Engelberg, 1965; Siskin et al., 1965). However, these occurrences are very dependent on the cell line considered. For example, incubation of human diploid fibroblasts (Enninga et al., 1984) or of a mouse leukemic cell line (Watanabe and Okada, 1967) at 31°C induced cell cycle arrest in G1/G0 phase; on the other hand, when HeLa cells were incubated at sub-normal temperatures there was an accumulation of cells in the M phase (mitosis) (Rao and Engelberg, 1965). In this work, we report on the effect of the cell cycle on AdV production. First, in order to establish the best cell cycle phase for AdV infection, 293 cells were chemically synchronised. Then, due to the inherent problems associated with the use of these chemicals compounds for production purposes, a synchronisation strategy based on temperature shift was developed and evaluated in terms of AdV production at high cell densities in 2 L bioreactors.

## 2. MATERIALS AND METHODS

### 2.1. Cell line and medium

Anchorage-dependent 293 cells, purchased from Stratagene (Catalog #240085), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated (56 °C, 30 min.) Foetal Bovine Serum (FBS) (all from Invitrogen, Glasgow, UK) and 4.5 g/L of glucose (Merck, Darmstadt, Germany) at a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. 293 cells were routinely propagated twice a week after trypsin/EDTA (Invitrogen, Glasgow, UK) addition at room temperature.

Suspension adapted 293 cells were grown in CD 293 (Invitrogen, Glasgow, UK), supplemented with 4 mM of glutamate (non-ammoniagenic medium) (Sigma-Aldrich, St. Louis, MO) at a humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C in shake flasks (Corning, NY). Cells were routinely propagated twice a week using an inoculum of  $0.5 \times 10^6$  cell/ml.

## 2.2. Adenoviral Vector

Recombinant AdV expressing the GFP protein were generated by homologous recombination of plasmid and digested DNA in E. coli strain BJ5183 (rec<sup>+</sup>). The GFP shuttle vector was first cloned into a transfer vector; the resulting plasmid was then linearized with a restriction enzyme (Swa I) and co-transformed into E. coli together with the plasmid containing AdV (pKP1.3). Recombinants were selected with ampicillin and screened by restriction enzyme analysis. The recombinant AdV construct was subsequently cleaved with Pac I to expose its ITR (Inverted Terminal Repeats) and transfected into 293 cells to produce viral particles.

## 2.3. Chemical synchronisation of 293 cells

For chemical synchronisation, 293 cells were seeded in 6 well plates at  $1 \times 10^6$  cell/well, with a culture volume of 2 mL of DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic solution (10000 µg/mL streptomycin and 10000 U/mL penicillin) (Sigma-Aldrich, St. Louis, MO). One day after, seeded cells were incubated with each inhibitor at three different concentrations during 6, 12, 24 and 48h. A control for each inhibitor where only the respective solvent was added to the cell culture was performed. The experimental design is summarised in Table 4.2.1. After the incubation period, 293 cells were digested at room temperature with trypsin/EDTA and analysed by flow cytometry.

Table 4.2.1. Reversible chemical inhibitors used and correspondent cell cycle arrest point. Stock solutions, tested concentrations and control experiments are shown for each case.

Inhibitor Type	Target Arrest Point	Inhibitor Stock solution	Tested concentrations	Control
Mimosine	Late G1	100 mM in a PBS	1 mM	PBS 0.1%
		solution 0.1%	1 $\mu$ M	(v/v) in
		(v/v) in DMSO	1 nM	DMSO
Hydroxyurea	G1/S transition	100 mM in a PBS	1 mM	PBS 0.1%
		solution 0.1%	1 $\mu$ M	(v/v) in
		(v/v) in DMSO	1 nM	DMSO
Aphidicolin	G1/S transition	2.96 mM in DMSO	0.1 mM	DMSO
			1 $\mu$ M	
			1 nM	
Staurosporine	G2	0.214 mM in ethanol 96% (v/v)	10 mM	Ethanol 96% (v/v)
			1 $\mu$ M	
			0.1 $\mu$ M	
Nocodazole	G2/M transition	13.3 mM in a PBS	1 mM	PBS 0.1%
		solution 0.1%	1 $\mu$ M	(v/v) in
		(v/v) in DMSO	1 nM	DMSO

#### 2.4. Infection of chemically synchronised 293 cells

Infection of chemically synchronised cells was performed by seeding 293 cells in 6 well plates at  $1 \times 10^6$  cell/well, with a culture volume of 2 mL of DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic solution (10000  $\mu$ g/mL streptomycin and 10000 U/mL penicillin). One day after, the best inhibitory conditions, determined beforehand, were added: 1 mM hydroxyurea during 48h, 0.1 mM aphidicolin during 12h and 1  $\mu$ M staurosporine during 48h. In the experimental controls only the solvents were added. After the incubation period, cells were centrifuged at 200 g during 10 min. at 4°C and suspended in 500  $\mu$ L of fresh DMEM without FBS



and reseeded in a new well. Then, cells were infected with AdV using a multiplicity of infection (MOI) of 10. 30 min. afterwards, 1 mL of DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic solution (10000 µg/mL streptomycin and 10000 U/mL penicillin) was added to each well. Infected 293 cells were then harvested at several times after digestion at room temperature with trypsin/EDTA.

## 2.5. Temperature Synchronisation of 293 cells

For temperature synchronisation, 293 cells adapted to grow in suspension were inoculated at  $0.5 \times 10^6$  cell/ml in 125 mL shake flasks with a working volume of 40 mL in a humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C. Once the concentration of  $2 \times 10^6$  cell/ml cells was reached, incubation at 29, 31, 33, 35 e 37°C was carried out for 72h, and the cell cycle followed by flow cytometry.

## 2.6. Infection of 293 cells synchronised by temperature shift

Infection of 293 cells synchronised by temperature shift was done in 2 L bioreactors (Braun, Melsungen, Germany). For this purpose, two bioreactors were inoculated at  $0.5 \times 10^6$  cell/ml with 293 cells adapted to grow in suspension. Once the population reached the concentration of  $3 \times 10^6$  cell/ml, one of the bioreactors (control) was infected and the other was synchronised at the S phase of the cell cycle and infected. All the infections were done using a MOI of 10 and AdV harvested at 48 hpi. Synchronisation was performed by firstly lowering the culture temperature to 31°C during 67h and then restoring it to 37°C during 72h. The agitation rate was maintained at 110 rpm ( $N_{Re} = 6800$ ); pH was controlled at 7.2 by aeration with a CO<sub>2</sub> gas-mixture and NaOH 0.2 M; the dissolved oxygen was controlled at 80% air saturation.

## 2.7. Flow cytometric analysis

To quantify the cell-cycle distribution, cells were stained with propidium iodide to measure the DNA content by flow-cytometric analysis (FACS). Cells were centrifuged at 200 g for 10 min. at 4°C, washed with ice-cold PBS and stained with ice-cold Vindelov's solution (Vindelov et al., 1983): 1 g/l trisodic citrate, pH 7.6; 50 mg/l propidium iodide (all from Sigma-Aldrich, St. Louis, MO); 0.1% NP 40 (Roche Diagnostics, Germany); 700 U/l RNase A (Sigma-Aldrich, St. Louis, MO); 0.01 M NaCl (Merck, Darmstadt, Germany) for a final cell concentration of  $1 \times 10^6$  cell/ml. All FACS analyses were performed on a CyFlow<sup>®</sup> Space (Partec, Germany) equipped with a blue solid state laser set at 488 nm. Data analysis was performed using FlowMax<sup>®</sup> software (Partec, Germany).

## 2.8. Virus culture samples preparation

Infected 293 cells were harvested at 48 hpi and centrifuged at 1000 g for 10 min. at 4°C. The resulting cell supernatant was distributed into small aliquots and stored at -85°C for titration of extracellular virus. The resulting cell pellet was resuspended in a known volume of 10 mM Tris buffer, pH 8.0 supplemented with 2 mM MgCl<sub>2</sub> (Merck, Darmstadt, Germany) and 0.1% (v/v) Triton (Sigma-Aldrich, St. Louis, MO). Cells were disrupted by vortexing for 1 min. and cell debris removed by centrifugation at 3000 g for 10 min. at 4°C. The resulting cell lysate supernatant was dispensed into small aliquots and stored at -85°C for quantification of intracellular viral content.

## 2.9. Adenoviral vector titration

AdV titration was done using anchorage-dependent 293 cells seeded in 24 well plates at  $0.25 \times 10^6$  cell/ml. One day later, seeded cells were counted

by haemocytometer and infected with 1 ml of serial dilutions of the AdV in infection medium (DMEM + 10% FBS). After 17 to 20 h of incubation 293 cells were then harvested after digestion at room temperature with trypsin/EDTA and immediately analysed in the flow cytometer. Flow cytometric data was acquired using a CyFlow<sup>®</sup> Space. The green fluorescence signal was collected by a photomultiplier tube after passing through a 525 ( $\pm$  20) nm band pass filter (FL1). GFP fluorescence of  $3 \times 10^4$  single viable cells per sample selected on FS versus SS scatter basis were analysed and high fluorescent cells were gated. The cytometric parameters were set to provide accurate discrimination between non-fluorescent negative cells and positive GFP-fluorescence cells on a FL1 versus FSC density plot in order to estimate the proportion of infected cells. Data analysis was performed using FlowMax<sup>®</sup> software.

#### 2.10. Cell concentration and viability determination

Cell concentration and viability were determined by counting cells on a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany) using the trypan blue (Invitrogen, Glasgow, UK) dye exclusion method.

### 3. RESULTS AND DISCUSSION

#### 3.1. 293 cell synchronisation with chemical compounds

To synchronise 293 cells at each phase of the cell cycle, 293 cells were incubated with different cell cycle inhibitors (mimosine, hydroxyurea, aphidicolin, staurosporine and nocodazol) at different concentrations (Table 4.2.1). As shown in Figure 4.2.1, the higher synchronisation was obtained: i) in the G1 phase (81% of the population) by incubation of 1 mM hydroxyurea during 48h; ii) in the S phase (47% of the population) by incubation of 0.1 mM aphidicolin during 12h; and iii) in the G2 phase (66%

of the population) by incubation of 1  $\mu\text{M}$  staurosporine during 48h. For the other inhibitors tested, mimosine and nocodazol, no synchronisation was obtained at the concentrations and incubation periods tested, meaning that perhaps the concentrations tested were lower than the ones necessary to arrest cells; since synchronisation in each phase of the cell cycle had been achieved with the other 3 inhibitors, no further assays were performed with mimosine and nocodazol.

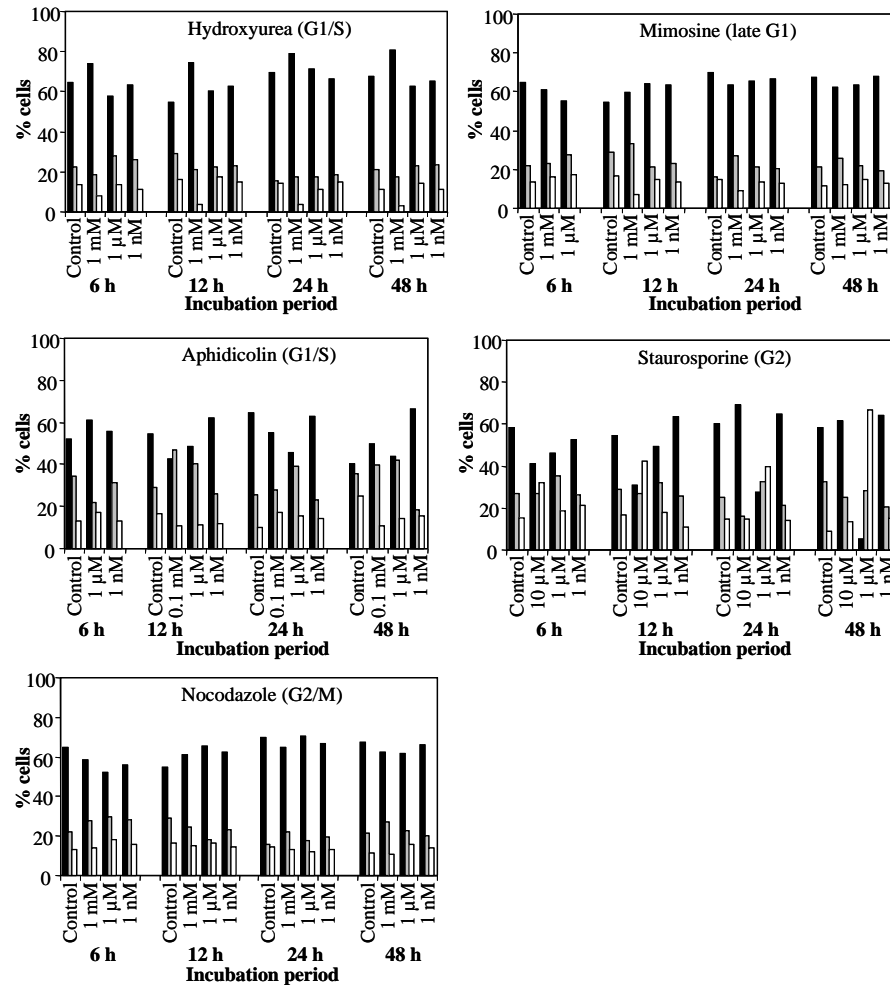


Figure 4.2.1. Proportion of 293 cells in the G1 (■), S (▒) and G2 (□) phases of the cell cycle, after incubation with the specified cell cycle inhibitor during 6, 12, 24 and 48h.

Despite the fact that aphidicolin was reported to inhibit the cell cycle in the G1/S phase transition (Jackson, 1995), our results show that this inhibition occurs during the S phase, possibly because even the highest aphidicolin concentration tested (0.1 mM) is still below the concentration needed for the complete inhibition of all the active DNA polymerase- $\alpha$ ; thus the cell cycle can pass through the G1/S phase transition and enter a longer lasting S phase since less DNA polymerase- $\alpha$  is active, resulting in an accumulation of cells in the S phase.

### 3.2. Adenovirus vector production in chemically synchronised 293 cells

Table 4.2.2: Cell concentration at the time of addition of inhibitors (Initial cell concentration) and after cell synchronisation (Final cell concentration). Control for each inhibitor was performed with addition of the respective solvent.

	Initial cell concentration ( $\times 10^6$ cell/ml)	Final cell concentration ( $\times 10^6$ cell/ml)
Hydroxyurea	1.2	1.0 <sup>1</sup>
Aphidicolin	1.0	0.63 <sup>2</sup>
Staurosporine	1.3	0.62 <sup>1</sup>
Hydroxyurea Control	1.0	2.3 <sup>1</sup>
Aphidicolin Control	1.0	1.5 <sup>2</sup>
Staurosporine Control	1.0	2.4 <sup>1</sup>

<sup>1</sup>Cell concentration determined 48h after inhibitor/solvent addition.

<sup>2</sup>Cell concentration determined 12h after inhibitor/solvent addition.

To define the optimal cell cycle phase for AdV infection, AdV productivity using those chemically synchronised 293 cells was evaluated by infecting in each phase of the cell cycle with a MOI of 10, after medium exchange that was carried out to remove the inhibitor. Despite the fact that synchronisation decreases cell viability (Table 4.2.2), the results obtained

(Figure 4.2.2) show that the highest cell specific productivity was obtained when aphidicolin (S phase synchronisation) was used, a 2.6 fold increase on cell specific productivity being obtained when compared to the non-synchronised control population. On the other hand, the use of staurosporine (G2 phase synchronisation) resulted in an 11 fold decrease on cell specific productivity while the use of hydroxyurea (G1 phase synchronisation) gave rise to a 17 fold decrease, when compared to the non-synchronised control population. These results confirm our earlier reported hypothesis (Zhang et al., 2006), that the S phase of the cell cycle is the optimal phase for AdV infection for increased productivity; this also substantiates the observed cell cycle progression towards S phase induced by Ad infection described by Berk (2005).

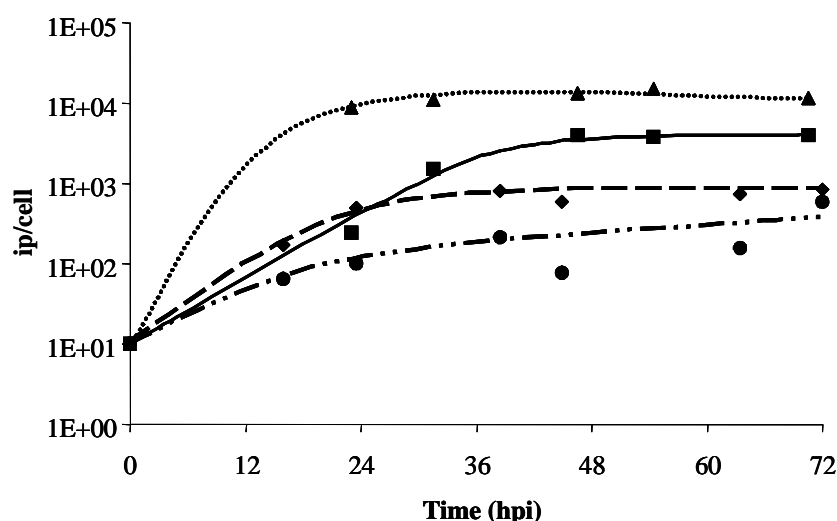


Figure 4.2.2. Profiles of cell specific productivity of chemically synchronised 293 cells in G1 phase (hydroxyurea) (circles), S phase (aphidicolin) (triangles) and G2 phase (staurosporine) (diamonds), and without synchronisation (control) (squares). Inhibitors were removed immediately before infection at a MOI of 10.

## 3.3. Model for adenovirus production in chemically synchronised cells

In order to develop a model that can predict AdV productivities with different amounts of cells at the S phase of the cell cycle, cell specific productivity was plotted against the amount of cells at G1, G2 and S phases of the cell cycle (Figure 4.2.3).

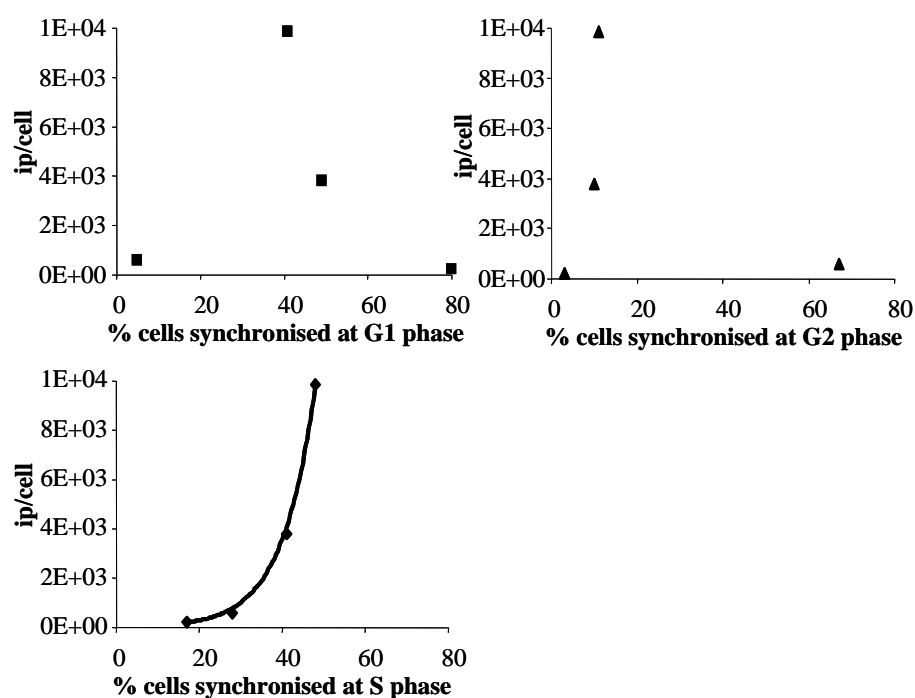


Figure 4.2.3. Profiles of cell specific productivity of chemically synchronised 293 cells in G1 phase (squares), G2 phase (triangles) and S phase (diamonds) of the cell cycle.

As can be seen in Figure 4.2.3, it is clear that only the amount of cells at the S phase of the cell cycle directly influences AdV productivity. The effect of the other two phases, G1 and G2, are inconsistent in terms of AdV productivity allowing the conclusion that increased amounts of cells in either G1 or G2 phases does not influence AdV productivity. Conversely, an

exponential relationship between the cell specific productivity and the amount of cells at the S phase of the cell cycle exists, for which description the following equation was considered:

$$dV/dS = kV \quad (1)$$

where V corresponds to the cell specific productivity, S to the amount of cells at the S phase of the cell cycle and k is a constant, virus produced per amount of synchronised cells. By interpreting equation (1) is possible to obtain:

$$\ln(V) = \ln(V_0) + kS \quad (2)$$

By fitting the experimentally obtained data it is possible to determine  $\ln(V_0)$  (3.0432) and k (0.1266). Thus, the experimental correlation can be represented in Figure 4.2.4.

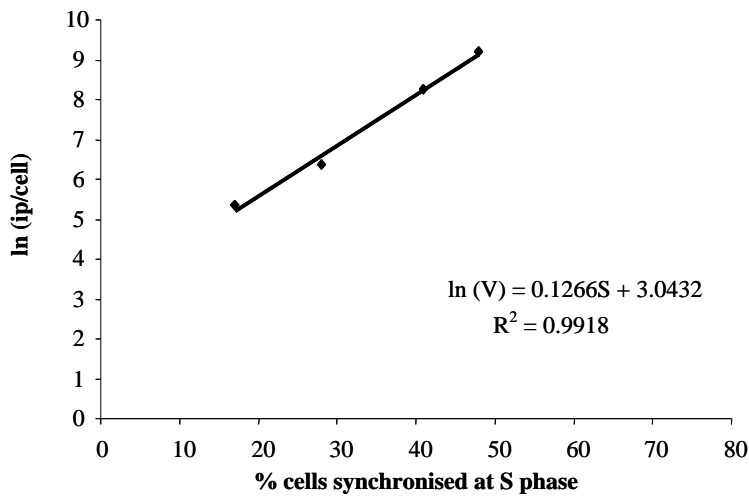


Figure 4.2.4. Correlation between cell specific productivity and amount of cells at the S phase of the cell cycle based on equation (2).



## 3.4. 293 cell synchronisation by temperature shift

Having established S phase as the optimal phase for AdV infection in terms of cell specific productivity and, since chemical synchronisation decreases cell viability and is costly for large scale production purposes, temperature synchronisation was also evaluated. To achieve cell cycle arrest in G1/G0 phase, 293 cells were incubated at 29°C, 31°C, 33°C and 35°C and compared with a control at 37°C. As shown in Table 4.2.3, the only incubation temperature where cell death occurred was 29°C; for all other temperatures tested specific growth rates ranged from 2.2 (at 35°C) to 12.6 (at 31°C) times less than the ones verified in the control (37°C), which in fact was expected since the optimal growth temperature for most mammalian cell lines is between 35 and 37°C (Rieder and Cole, 2002).

Table 4.2.3: Specific growth rates obtained during a 72h incubation period at sub-optimal temperatures.

Temperature (°C)	Specific growth rate: $\mu$ ( $\text{h}^{-1}$ )
29	-0.0018
31	0.0011
33	0.0050
35	0.0062
37	0.0139

Concerning cell cycle synchronisation, the results (Figure 4.2.5) show that the highest percentage of cells at the G1/G0 phase occurred at 31°C and 67 h of incubation, with a maximal synchronisation of 77%, representing a 1.3 fold increase compared with the control for the corresponding period of time. To check whether a higher incubation period at 31°C could increase the proportion of cells arrested in G1/G0 phase, cells were incubated during 100h at 31°C, however no further increase was observed (data not shown).

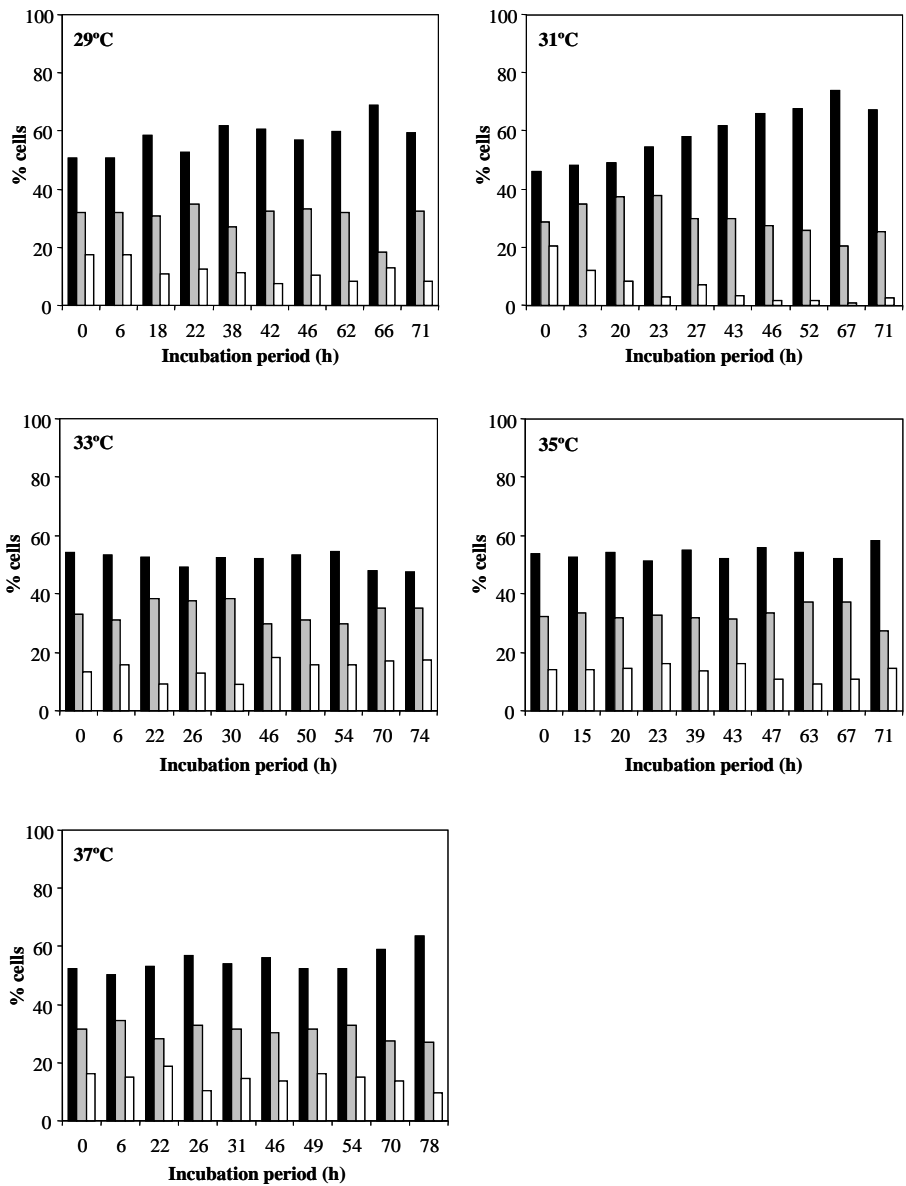


Figure 4.2.5. Effect of incubation temperature on the proportion of 293 cells in the G1 (■), S (■) and G2 (□) phase of the cell cycle.

Possible explanations for this observation can be the induction of p21, an inhibitor of the G1/S transition synthesis, reported for glioblastoma human

cells after a 4°C cold shock during 1h (Ohnishi et al., 1998). Furthermore, studies with human or mouse embryonic fibroblasts incubated at 28°C have shown increased level of p53 (a transcription factor that blocks cell cycle at G1/S transition in case of damaged DNA) and p21, resulting in cell cycle arrest at G1/S transition; this does not take place in embryonic cells of p53 knockout mice submitted to the same conditions (Matijasevic et al., 1998). All these studies indicate that cell cycle arrest in mammalian cells in response to cold shock is induced by the activation of pathways that regulate cell cycle checkpoints, as the ones mediated by p53. Once activated, this pathway induces synthesis of p21 which blocks G1/S transition by inhibition of cyclin dependent kinases (CDKs). This blockage persists until the p21 activity decays below a threshold value, after which cell cycle resumes.

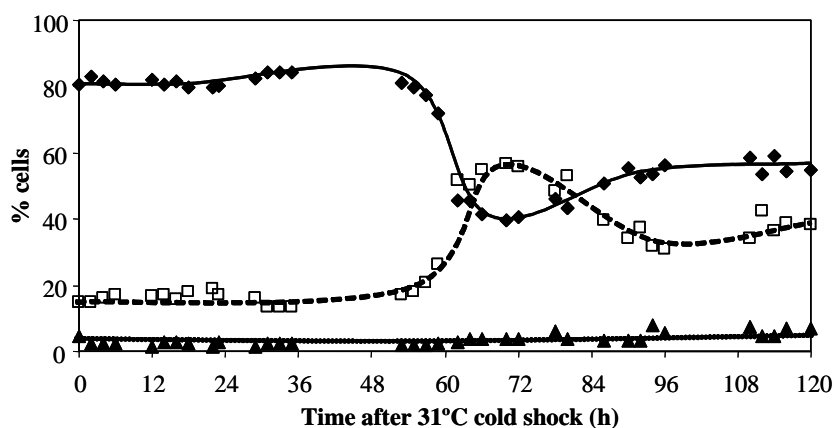


Figure 4.2.6. Cell cycle progression of 293 cells during 120h at 37°C after a 67h cold shock at 31°C. G1 phase is represented by (◆), the S phase by (□) and the G2 phase by (▲).

As the aim was to obtain cell synchronisation in S phase, the cell cycle progression of 293 cells incubated at 37°C, after a 67h cold shock at 31°C, was followed. As shown in Figure 4.2.6, cell cycle synchronisation in G1

phase is maintained up to 53h after the cold shock. Thereafter, an increase in the proportion of cells in S phase of the cell cycle can be observed, a maximum of 57% being obtained at 72h.

### 3.5. Adenovirus vector production in synchronised 293 cells by temperature shift

The effect on AdV production of this temperature synchronisation in the S phase at high cell densities was tested in 2 L bioreactors, where 293 cells were infected after S phase synchronisation was reached by a temperature strategy of 67h at 31°C followed by 72h at 37°C. A control bioreactor, without temperature shift, was also done at 37°C. In both cases, cells were grown up to  $3 \times 10^6$  cell/mL and infected immediately (control) or after synchronisation. It was observed that at these high cell densities the 67h cold shock at 31°C decreased the cell concentration from 3 to  $1.6 \times 10^6$  cell/mL (data not shown). Nevertheless, a significant increase in the percentage of cells at the S phase of the cell cycle was obtained for this smaller cell population.

Table 4.2.4: Effect of 293 cell cycle synchronisation in S phase, by temperature shift, on cell specific productivity at a cell concentration at infection of  $3 \times 10^6$  cell/mL.

	cell specific productivity (ip/cell)	AdV volumetric productivity (ip/mL)
Control	$3.0 \times 10^3$	$8.4 \times 10^9$
S phase synchronised cells	$2.2 \times 10^4$	$3.5 \times 10^{10}$

Infections were performed at MOI of 10 and AdV harvested at 48 hpi.

As shown in Table 4.2.4, a 7.3 fold increase on cell specific productivity ( $2.2 \times 10^4$  ip/cell) was obtained when cell cycle was synchronised in S phase

before infection, comparing to that obtained in the control bioreactor ( $3.0 \times 10^3$  ip/cell); although cell viability decreased almost by half after synchronisation, this improvement on cell specific productivity results in an overall 4 fold increase upon AdV volumetric productivity. Curiously, this increase on AdV productivity was obtained for an increase of only 1.7 times on the percentage of cells at the S phase of the cell cycle. Seidman et al. (2001) reported that both the high-affinity coxsackie-adenovirus receptor (CAR) for Ad fiber protein and the low-affinity  $\alpha_v$  integrin receptor for Ad penton base protein showed increased cell surface expression at G2/M phase (1.5 fold and 2 to 3 fold increase respectively) when comparing with the G1 phase of the cell cycle. Although no results were shown for cells synchronised at the S phase of the cell cycle, it is expected that these Ad receptors also reach increased values at this phase when compared with the G1 phase of the cell cycle. This can explain the results obtained since, in addition to an increased efficiency of S phase synchronised cells to support AdV production, AdV infection efficiency increases in the S phase synchronised cells.

Finally, by using the model developed previously, for a percentage of cells at the S phase of the cell cycle of 57% it was expected a cell specific productivity of  $2.8 \times 10^4$  ip/cell, 21% more than the obtained. This different may be due to the fact that the amount of cells at the S phase of the cell cycle obtained with the temperature shift strategy is out of the range. In addition, this small difference can be due to the fact that the synchronisation method used was different.

#### 4. CONCLUSIONS

The results presented above confirmed that it is possible to obtain substantial cell cycle synchronisation for 293 cells by chemical means at G1 (81%), S (47%) and G2 (66%) phases (Figure 4.2.1); furthermore, these results prove that S phase is the optimal cell cycle phase for AdV infection

(Figure 4.2.2). In addition a mathematical model that can predict AdV productivities with S phase cell synchronisation was developed with success.

For large scale production purposes, a 293 cell cycle synchronisation in the S phase (57%) was also accomplished by incubating at 37°C during 72h after a 67h cold shock at 31°C (Figure 4.2.5 and 4.2.6). This strategy yield a 7.3 fold increase on cell specific productivity ( $2.2 \times 10^4$  ip/cell) compared to that obtained in a non-synchronised population ( $3.0 \times 10^3$  ip/cell) which had 23% less cells in the S phase than the synchronised population at infection time. A significant drop on cell viability was observed after the temperature shift treatment (around 50%); nevertheless, due to the large increase in cell specific productivity, this strategy allowed a 4 fold increase on AdV volumetric productivity.

These results confirm the physiological state of the cell as an important factor on AdV production at high cell densities and the S phase of the cell cycle as the best phase for AdV infection. In addition, the data presented here show that the temperature shift method can be used for S phase synchronisation without the use of chemical substances. Nevertheless, since the culture temperature affects such cellular events as growth, viability, protein synthesis and metabolism, further studies are needed to achieve an efficient process for AdV production.

## 5. ACKNOWLEDGEMENT

The authors are grateful to Marlene Carmo for all the support concerning the flow cytometric analyzes. The financial support received from the European Commission (Project RP/PPR ORALVAC ICA4-CT-2000-30027) and from Fundação para a Ciência e Tecnologia (FCT) - Portugal (Project POCTI/BIO/46515/2002, grant SFRH/BD/10614/2002 and grant SFRH/BPD/7147/2001) are also acknowledged.

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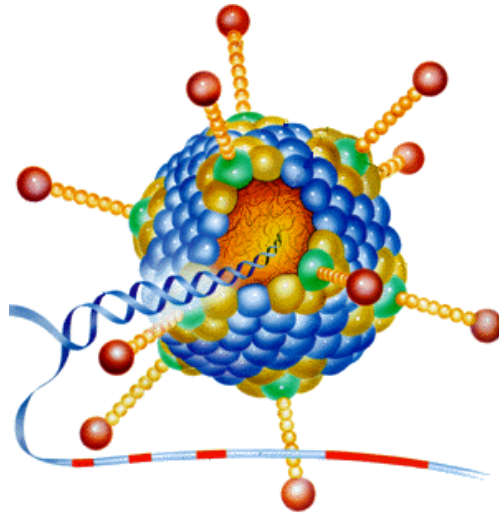
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## CHAPTER V

### Discussion and Conclusions



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## 1. DISCUSSION

For early gene therapy clinical trials a production below  $10^{15}$  virus particles per batch is suitable due to the relatively small number of patients involved, while some vaccine applications may require the development of larger amounts of virus particles; both cases require maximization of the AdV volumetric productivity. Both viral productivities, cell specific and volumetric, depend on the genetic AdV design and the cell line used to propagate the virus, as well as the medium formulation, scale and culture system used; the manipulation of these factors in process optimisation is required.

Almost all major media manufactures have commercialised specific serum-free formulations designed and optimised for the cultivation of AdV, such as the CD 293 medium from Invitrogen and EX-Cell medium from JRH, as mentioned in Chapter II. Such formulations allow cell growth to reach high cell densities of up to  $8 \times 10^6$  cell/ml in batch mode. However, cell specific productivity for virus production is dramatically reduced for densities higher than  $1 \times 10^6$  cell/ml. Indeed the medium that allows for a higher cell density does not necessarily allow for higher virus productivity, as observed in the case of EX-Cell and CD 293 media where the medium responsible for the higher cell specific productivity has the lowest cell density at the end of the batch growth phase; this is due to the fact that, most often media are designed and optimised for cell growth. As shown in Chapter III Part 1, during the virus production phase the specific consumption rates of several medium components (such as glucose and amino acids) were shown to increase leading to the exhaustion of some nutrients, such as threonine, glutamine and glucose, when infection was performed at CCI of  $3 \times 10^6$  cell/ml. Nevertheless the refeed of these nutrients did not allow for a significant improvement on AdV production at such cell density. On the other hand, the refeed of these nutrients, mainly glutamine at infection, led to an increase on ammonia production which effects upon AdV

production was unknown.

Most of the reported studies concerning the effect of ammonia upon cell growth and productivity are conducted after ammonia addition to the culture medium. However this experimental approach does not mimick the real cell behaviour as cell internal concentrations are different depending on where the ammonia is being produced by or added to the cells; thus, large amounts of ammonia were needed to observe any inhibitory effect. In this thesis, ammonia specific productivity was significantly decreased by adapting 293 cells to grow in CD 293 medium where glutamine was exchanged by glutamate. With this strategy, a prolonged lag phase, reduced growth rates and lower maximum cell densities were obtained. In addition, an increase in all amino acids specific consumption rate was also observed, particularly glutamate, which is produced in cells growing in glutamine supplemented media. On the other hand, no significant variations were observed in glucose specific consumption and lactate production rates in non-infected cells. By infecting these cells, at a CCI of  $3 \times 10^6$  cell/ml, an almost 2 times increase on AdV volumetric productivity was obtained, proving the importance of high ammonia concentration, as the ones observed at this CCI, on AdV production at high cell densities.

By comparing both ammoniagenic (glutamine supplemented) and non-ammoniagenic (glutamate supplemented) media in Chapter III Part 2, the mechanisms of ammonia inhibition of AdV production are discussed. Intracellular pH ( $pH_i$ ), observed to decrease with the increase on ammonia concentration during cell growth, was responsible for a series of phenomena that led to AdV losses at high cell densities when the ammoniagenic medium was used. These changes in the  $pH_i$  were observed to be enough to activate both an acidic pathway of apoptosis and an enzyme capable to cleave the AdV DNA (DNase II); thus, cell death started earlier and the amount of AdV DNA available for encapsulation decreased significantly explaining the reduced cell specific productivity obtained.

Although a significant improvement on cell specific productivity was

obtained by adapting the cells to non-ammoniagenic medium, this improvement is still far from optimal, since the “ideal” situation would have been a three fold increase at CCI of  $3 \times 10^6$  cell/ml. Therefore, in Chapter IV, the physiological state of the cells at the time of infection was evaluated by examining the cell cycle distribution at different cell densities. The maximum amount of cells at the S phase of the cells cycle was found around  $1 \times 10^6$  cell/ml (the optimal concentration for AdV infection) and decreased with increasing cell density. By chemically synchronising the cells at each phase of the cell cycle we observed that the cell specific productivity was significantly increased when cells were infected after synchronisation at the S phase of the cell cycle. On the other hand, when cells were infected after synchronisation in either G2 or G1 a significant decrease in cell specific productivity was obtained, more pronounced for G1. These results clearly confirm S phase of the cell cycle as the optimal phase for AdV infection and support our previous hypothesis that the cell physiology at the time of infection is one critical parameter on the “cell density effect”. From these results the development of a simple method for cell synchronisation avoiding the use of chemical inhibitors would be instrumental. This was accomplished by using a temperature shift strategy, consisting in decreasing the temperature to 31°C during 67 h and increasing it again to 37°C during 72 h. As a result, 57% of the cells were synchronised at the S phase of the cell cycle, an increase of 23% when compared with non-synchronised cells. Although a drop on cell viability was observed, a 7.3 fold increase on cell specific productivity was obtained, corresponding to a 4 fold increase on AdV volumetric productivity when compared with infection of cells in the non-ammoniagenic medium at a CCI of  $3 \times 10^6$  cell/ml.

## 2. CONCLUSION AND FUTURE WORK

This thesis aimed to overcome the limiting factors on AdV production at



high cell densities, an issue which had been ongoing in the literature for a while. Two factors were found to play an important role on AdV production at high cell densities: ammonia and cell cycle. High concentrations of ammonia, as those observed at CCI of  $3 \times 10^6$  cell/ml, were shown to induce cytoplasmatic acidification leading to cell death and AdV DNA degradation, leading to a decrease on the bioactive AdV production. On the other hand, the percentage of cells in the S phase of the cell cycle was observed to decrease with increasing cell density with a maximum at approximately  $1 \times 10^6$  cell/ml. The S phase was instrumental to improve AdV production at the time of infection and a substantial increase on cell specific productivity was obtained when infection took place after a substantial cell population synchronised in this phase. In addition, since the percentage of synchronised cells at the S phase of the cell cycle is higher at CCI of  $3 \times 10^6$  cell/ml than the ones observed at CCI of  $1 \times 10^6$  cell/ml, the cell specific productivity increased 4.8 times, a 7.6 fold increase being obtained in terms of AdV volumetric productivity.

Future work will focus on the optimisation of S phase synchronisation. The maintenance of cell viability during infection at high cell densities in S phase of the cell cycle synchronised cells will led to considerably increased AdV titers. Temperature changes would affect such cellular events as growth, viability, protein synthesis and metabolism. Thus, in order to achieve an efficient process for AdV production, cell synchronisation needs to be further improved.

### 3. SCIENTIFIC CHALLENGES ON AdV AS VACCINES

The major potential drawbacks for the use of AdV as a veterinary vaccine is the rare but possible emergence of E1 containing vectors, due to eventual minute homologous recombination events between the viral E1-containing (permissive) mammalian host cell genome and the virus itself, restoring the E1 gene to the viral genome and originating recombinant competent

adenovirus (RCA). Its appearance in batches is a chance process and is therefore unpredictable and difficult to control. This is a significant issue for GMP manufacturing. It is also unwanted from a safety point of view, as upon coinfection of a cell, RCA causes the E1-deleted AdV to replicate in an uncontrollable way. In addition, RCA has been shown to cause inflammatory responses. Therefore, RCA generation during production of E1-deleted AdV has to be circumvented. To reduce or eliminate the problem of RCA, an E1-transformed human cell line, PER.C6, was constructed (Fallaux et al., 1998) by transformation of primary human embryonic retinoblasts with limited homology with the viral sequences, thus reducing or eventually eliminating the emergence of RCA during virus amplification. Nevertheless, the licenses requested for using the PER.C6 technology makes its use improbable for veterinary vaccine production.

The immune response to AdV or its transgenes is a limiting factor in the successful application of these vectors. Antivector immune responses can be broadly divided into cellular and humoral. While the cellular immune response limits duration of transgene expression, the humoral response may reduce the therapeutic efficacy of repeated vector administration. It is also emerging that the humoral immune response to viral vectors is not limited to responses against the viral vector components but also includes anti-transgene responses that can affect the persistence of transgene expression. One approach to enhance Ad-mediated gene transfer is to modulate the host immune response by immunosuppression of the recipient organism such as cytokine treatment, T-cell depletion strategies and immunosuppressive drugs. Vectors with capsid components modified at specific capsid sites known to be targets for the anti-Ad-neutralizing antibodies might be useful in overcoming pre-existing, serotype-specific anti-Ad immunity. In addition, administration of different-serotype AdV, such as Ad 35, or different-species AdV, such as canine AdV, may circumvent the local host immunity elicited by a first vector administration. There are more than 100 different Ads isolated from man,

sheep, cattle, dog, horse, bird, etc.

Recent efforts to better characterize the *in vivo* host responses and barriers to Ad infection have defined a number of hurdles to the use of AdV for both gene therapy and vaccination. With this groundwork laid, systematic exploration of these barriers will hopefully lead to development in the means to circumvent them. This is already a very active area of research and is likely to continue to be productive.

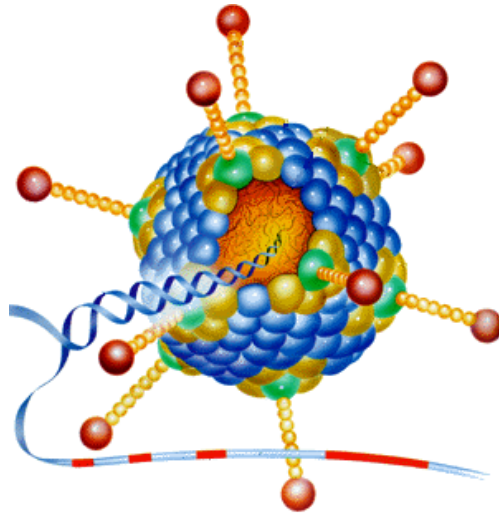
Up to now, many viruses have been used as viral vectors for delivery of foreign antigens (see Yokoyama et al. (1997) for a review). The development of these recombinant vaccines may be of value in the veterinary field, especially when attenuation of the target pathogen is not possible, when immunity to one or more pathogens must be raised at the same time or when satisfactory quantities of the target pathogen or the live vaccine cannot be produced easily or safely. For their practical use, potential safety issues must be overcome, such as reversion of virulence or recombination with field-type viruses spread in the environment and genetic stability. The selection of a good viral vector as a recombinant viral vaccine should be determined by the host range of the vector, safety consideration, replication properties, stability, the amount of foreign DNA which can be inserted and the place where the primary immune reaction is induced by antigens expressed by the vector.

Exciting advances in biotechnology are enabling the development of new vaccines with enormous possibilities that were once considered unthinkable. These newer vaccine approaches have benefited from an improved basic understanding of the immunology of antigen presentation and the factors important in sustaining memory immune responses. The continued improvement in our understanding of the nature of protective immunity against various infectious diseases, such as HIV and malaria, should allow the rational design of vaccines and immunization strategies that generate protective immune responses against infectious diseases for which no vaccines yet exist.

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ANNEXES



## ANNEX A

Effect of MOI and medium composition on adenovirus infection kinetics

Ferreira T.B., Alves P.M., Gonçalves D., Carrondo M.J.T. (2005) in Gòdia F. and Fussenegger M. (Ed), *Animal Cell Technology Meets Genomics*, Springer, 329-332.

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## 1. INTRODUCTION

Recombinant adenoviral vectors (AdV) are highly efficient for gene transfer in a broad spectrum of cell types and had become one of the vectors of choice for delivery and expression of foreign proteins for gene therapy and vaccination purposes. The market requirements for AdV are constantly increasing causing a high demand of methodologies for the production in large scale of concentrated vectors.

The importance of infection kinetics on viral and protein production and the significance of variables such as MOI (Multiplicity Of Infection) and HPI (Hours Post Infection) in process optimisation have been demonstrated in previous studies with other viral/eukaryotic expression systems e.g. baculovirus-insect cells (Cruz et al., 1999), reinforcing that an optimized strategy of MOI and HPI is mandatory to increase production yields. Other important parameter affecting the viral infection process is serum concentration in the culture media (Petricevich et al., 2001; Maranga et al., 2002).

In this work, AdV production kinetics was investigated. Factors such as medium composition, serum content, MOI, and harvest time were studied. A first generation recombinant adenovirus type 5 was used.

## 2. MATERIALS AND METHODS

**Cell Growth:** 293 cells (ATCC-CRL-1573), were grown in MEM supplemented with 2 mM glutamine and 5% (v/v) FBS (all from Gibco, UK), at a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C or in CD 293 (a serum and protein-free medium Invitrogen, UK) supplemented with 2 mM glutamine at a humidified atmosphere of 8% CO<sub>2</sub> in air at 37°C; **AdV Quantification:** AdV titration was performed by the end-point dilution method (TCID<sub>50</sub>) using 96 well plates and 293 cells. The titer was calculated according to the method of Reed

and Muench (Summers and Smith 1987). Virus titer were normalized to the maximum one in the experiment.

### 3. RESULTS AND DISCUSSION

#### 3.2.1. Effect of MOI on AdV Production

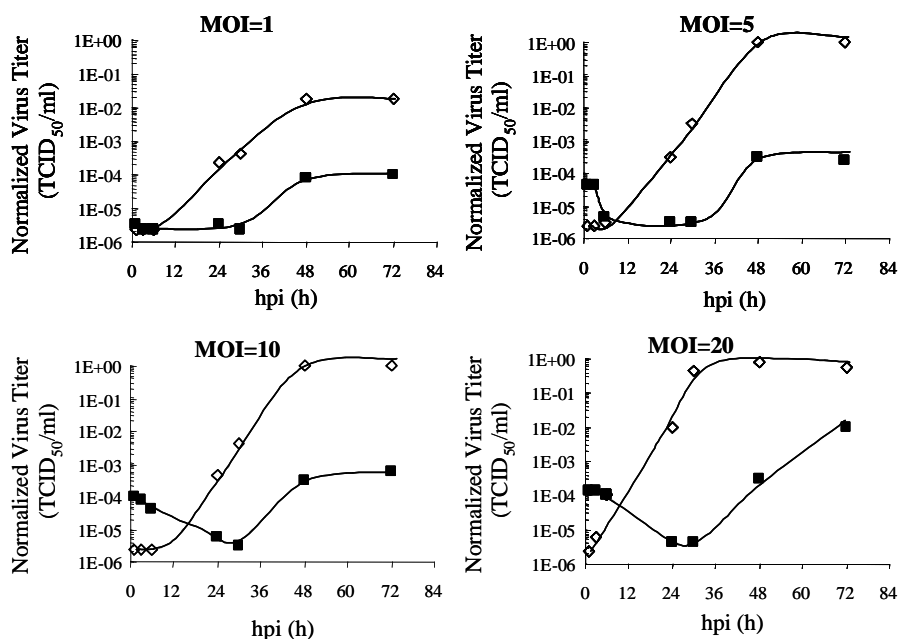


Figure A.1. Effect of MOI on normalized virus titer ( $\diamond$  intracellular,  $\blacksquare$  extracellular).

To evaluate the effect of MOI on AdV production, 293 cells were infected with a MOI of 1, 5, 10 and 20. The AdV production profiles are shown in Figure A.1. The maximum vector titer increased by a factor of  $10^2$  when the MOI was increased from 1 to 5. Further increase in MOI did not affect vector titer but caused a shift of the optimal harvest time; taking into account the AdV net output ( $\text{AdV}_{\text{Out}}/\text{AdV}_{\text{In}}$ ) it is clear that the best yields are obtained when a MOI of 5 is used. The profiles of AdV release into the

supernatant (due to cell lysis) were similar for MOI of 1, 5 and 10. The results show that for 36 hpi the amount of AdV released into the supernatant is not significant as compared with the intracellular content.

### 3.2.2. Effect of Serum on Cell Growth and AdV Production

In order to understand the effect of serum concentration on AdV production, cells were grown in MEM supplemented with 10% FBS and at the time of infection (MOI 5) the total volume of the culture medium was replaced by MEM supplemented with 10, 5, 1 and 0% of FBS. AdV were harvested at 48 hpi. The highest AdV yields were obtained in MEM supplemented with 5% FBS (Figure A.2).

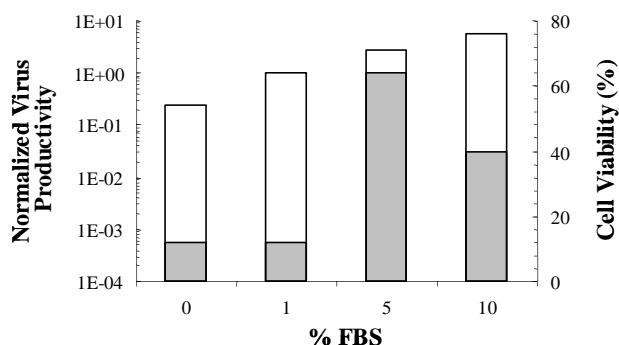


Figure A.2. Normalized virus productivity (■) and cell viability (□) at different serum concentration for a MOI of 5 at 48 hpi.

AdV production decreased by a factor of  $10^3$  when 0 and 1% FBS concentrations were used indicating the importance of serum on viral infection as extensively reported in the literature (Petricevich et al., 2001; Maranga et al., 2002). Interestingly a decrease on AdV production was observed for 10% FBS containing medium suggesting that, when in excess, some serum components (e.g. proteins) can compete with AdV for cell

receptors by blocking viral receptors and consequently decreasing the probability of viral entrance into the cell. Also, a concomitant decrease in cell viability was observed with the decrease in serum content (Figure A.2). However, supplementing cell culture media with serum presents several drawbacks as mentioned before. Recently, several serum and protein free culture media became commercially available, as an alternative approach to serum containing medium formulations. Thus, in order to avoid the use of serum, CD 293, a serum and protein free medium, was compared with MEM supplemented with 5% FBS in their ability to grow 293 cells in T-Flask, since 293 cells were not able to grow in suspension with MEM medium (data not shown). As shown in Figure A.3, CD 293 presented the highest specific growth rate and allowed the highest maximum cell number, being a good alternative to the serum supplemented media in terms of cell growth.

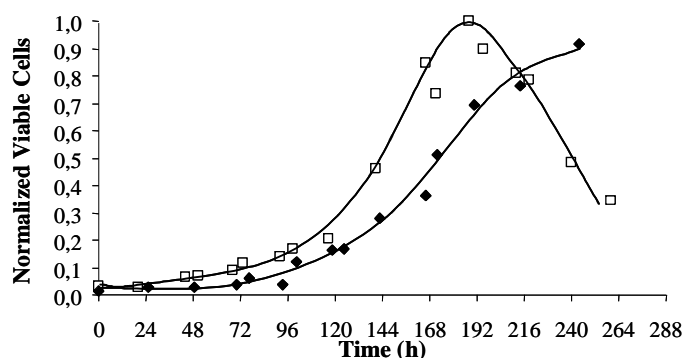


Figure A.3. Growth curves of normalized 293 cells obtained in two different culture media: (◆) MEM + 5% FBS and (□) CD 293.

To evaluate the performance of both media to produce AdV in T-Flasks, cells were grown in MEM supplemented with 5% FBS or CD 293 and infected at confluency around 90% with a MOI of 5 after media exchange. The results are presented in Figure A.4. The highest AdV yields were obtained in CD 293 medium confirming the potentiality of this media to “support”

AdV production. The fact that CD 293 is a protein free medium could explain the higher AdV productivity obtained, since components that may compete with AdV for cell receptors are not present.

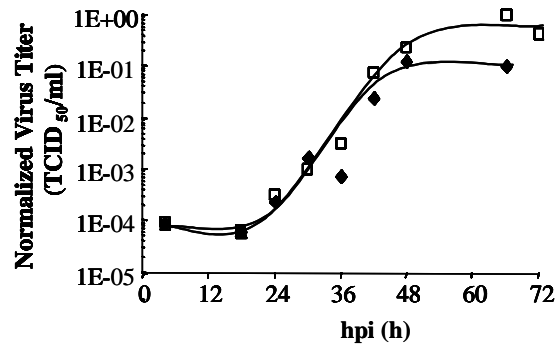


Figure A.4. Normalized virus titer at a MOI of 5 with (□) CD 293 and (♦) MEM+5% FBS.

#### 4. CONCLUSIONS

The best AdV production yields were obtained for a MOI of 5 at 48 HPI. Serum concentration affected AdV production yields and also cell viability. The best FBS concentration was 5%. Surprisingly, AdV infection seems to be hindered by the presence of a higher serum concentration (10%). CD 293, a serum and protein free medium, prove its potentiality as the best alternative for AdV production, not only because it supports higher AdV yields but also because it is possible to simplify the downstream process, since virus and prions contamination are avoid in the bioprocess.

#### 5. ACKNOWLEDGEMENT

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acknowledge the financial support received from the European Commission (Project ICF599A4PR01) and from Fundação para a Ciência e Tecnologia - Portugal (student grant SFRH/BD/10614/2002).

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## ANNEX B

Effect of vitamins or lipids addition on adenovirus production at high cell densities

Ferreira T.B., Carrondo M.J.T., Alves P.M. (2006) in Smith R. (Ed), Cell Technology Processes for Cell Products, Springer (in press).

## Abstract

Methodologies for production of concentrated adenovirus vectors (AdV) with warranted purity and efficacy at low cost are needed as the market for AdV is expanding fast. However, production of AdV that maintain a high specific yield is limited to cell densities in the range of  $1 \times 10^6$  cell/ml; the nature of the factors for this remains unknown. The aim of this work is to study the effect of addition of vitamins or lipids at the time of infection at high cell densities in order to see if these nutrients are limiting. No improvement on AdV productivity was observed. Furthermore, a significant decrease on cell specific productivity after vitamin addition was observed.

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## 1. INTRODUCTION

AdV became one of the vectors of choice for delivery and expression of foreign proteins for gene therapy and vaccination purposes (Ferreira et al., 2005a). Nevertheless, the production of AdV is currently limited by the so-called “cell density effect” (Nadeau and Kamen 2003). Several approaches have been made in order to overcome this drop in cell specific productivity concomitant with increased cell concentration at infection (CCI): Garnier et al. (1994), demonstrated that medium replacement at infection and the addition of glucose at 24 hours post infection (hpi) together with periodical pH adjustments, allowed a sustained maximum specific productivity at  $1.6 \times 10^6$  cell/ml; whereas Nadeau et al. (1996), further improved this strategy by also adding essential amino acids at 24 hpi thereby stabilising volumetric productivity at cell densities above  $2 \times 10^6$  and below  $3 \times 10^6$  cell/ml. Such results hint at the existence of substrate limitation and/or byproduct inhibition at high cell densities. In our latest study, by adapting the cells to non-ammoniagenic medium, a 1.8 fold increase on AdV volumetric productivity at CCI  $3 \times 10^6$  cells/ml was obtained (Ferreira et al., 2005b), although the “ideal” situation would have been an increase of 3 times at this CCI; thus, ammonia is an important parameter to be considered for infection at high cell densities, but not the only one.

Future improvements to the culture process may come from analysis of other nutrients than the usual glucose, lactate, ammonia and amino acids. A cocktail of different nutrients are present in the culture media that may have an important impact on AdV production as vitamins, lipids, hormones and growth factors. Thus, in this work, addition of vitamins and lipids at the time of infection at high cell densities was evaluated in order to see if these nutrients are limiting the AdV production at high cell densities.

## 2. MATERIALS AND METHODS

Suspension adapted 293 cells (ATCC-CRL-1573) were grown and infected in non-ammoniagenic medium in a 2L bioreactor (Braun) as described in Ferreira et al. (2005b). For the fed-batch operation mode, Vitamins and Lipids cocktail solutions (all from Gibco) were used; AdV titration was performed by the end-point dilution method (TCID<sub>50</sub>) using 96 well plates and 293 cells. The titer was calculated according to the method of Spearman and Kraber, as described in Darling et al. (1998).

## 3. RESULTS AND DISCUSSION

Vitamins and lipids are important nutrients for cell growth. Moreover, lack of such nutrients may affect cell productivities. Thus, two different fed-batch operation modes were tested with addition of: (1) vitamins or (2) lipids cocktail at the time of infection at CCI  $3 \times 10^6$  cell/ml.

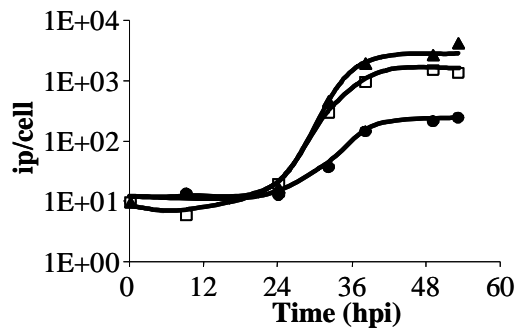


Figure B.1. Cell specific productivity after addition of vitamins (●) or lipids (□) at the time of infection, at CCI  $3 \times 10^6$  cell/ml. The control (▲) was performed without any nutrient addition.

As can be observed in Figure B.1, neither the addition of vitamins nor of lipids, at the time of infection, improved the cell specific productivity at this CCI. On the other hand, a significant cell specific productivity decrease

was observed after the addition of vitamins. Following the cell metabolism after infection it was possible to observe that none of the measured parameters was shown to be responsible for this significant decrease (Table B.1), suggesting that vitamins may have an effect on AdV stability.

Table B.1. Measured parameters during infection at CCI  $3 \times 10^6$  cell/ml concomitant with addition of vitamins.

Parameters	Result
Glucose	Not limiting
Lactate	Below inhibitory values
YLac/Glc	Unchanged
Amino acids	Not limiting
Ammonia	Below inhibitory values
Osmolality	Below inhibitory values
Cell growth	No effect

In order to evaluate the effect of vitamins on AdV stability, AdV were incubated with the vitamins cocktail at different concentrations during 48h at 37°C and pH 7.2 (Figure B.2). Although a small decrease on AdV titer was observed this was not statistically different from the ones in the control, meaning that vitamins have no influence upon AdV stability.

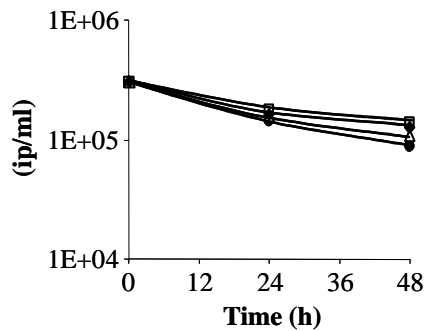


Figure B.2. AdV stability during incubation with medium supplemented with 0× (●) (control), 1× (□), 1.5× (Δ) and 2× (◆) of vitamins.

Several studies reported some vitamins as virus inactivators, e.g. riboflavin, present in our vitamins cocktail, was shown to selectively induce damage only to nucleic acids (Dardare and Platz 2002) and probably this may explain the significant decrease on cell specific productivity, since AdV DNA damage might be occurring during AdV replication.

In conclusion, from the lipids present in the cocktail no one seems to be limiting on AdV production at high cell densities, since no improvement on cell specific productivity was observed after addition of these nutrients. Concerning addition of vitamins an inhibitory effect appears to be present on AdV production after the addition of these nutrients.

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